Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen
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Elucidation of the role of the DFNA5 protein through the study in yeast and human cell lines

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Opheldering van de rol van het DFNA5 proteïne via modelsystemen in gist en humane cellen

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I. Samenvatting

Het *DFNA5* gen werd in 1997 gekarakteriseerd als een gen verantwoordelijk voor autosomaal, dominant, niet-syndromaal gehoorverlies (GV). Tot op heden zijn er reeds acht verschillende families geïdentificeerd met GV gerelateerd aan mutaties in *DFNA5*. Op genomisch niveau verschillen vier van de acht gevonden mutaties, maar ze resulteren telkens in *skipping* van exon 8 met een prematuur getrunceerd proteïne als gevolg. *Skipping* van exon 8 leidt tot een verschuiving van het *open reading frame*, resulterend in een verkorte versie van het wild-type niet-gemuteerde DFNA5-proteïne (wtDFNA5), genaamd mutant DFNA5 (mutDFNA5).

Behalve GV is wt*DFNA5* ook geassocieerd met verschillende vormen van kanker, zoals borst-, colorectaal-, maag- en melanoma kanker. In deze tumoren is wt*DFNA5* epigenetisch geïnactiveerd door hypermethylatie. Overexpressie van *DFNA5* in verschillende tumor cellijnen leidde echter tot een reductie in tumorvorming en koloniegrootte. Deze resultaten wijzen in de richting dat *DFNA5* een potentieel tumor suppressor gen is.

Gregan *et al.* waren de eerste die aantoonde dat mutant DFNA5 (mutDFNA5) een groeidefect induceerde in gist, maar de exacte rol van DFNA5 bleef voor lange tijd ongekend. Recent functioneel onderzoek in humane cellijnen echter, identificeerde DFNA5 als een apoptose-inducerend eiwit. Omdat reeds was aangetoond door Gregan *et al.* dat de DFNA5-celdood-gerelateerde mechanismen minstens gedeeltelijk zijn geconserveerd tussen humane cellijnen en gist, werd er in het eerste deel van deze thesis een nieuw *Saccharomyces cerevisiae* gistmodel ontwikkeld en gevalideerd door verschillende celdood assays om de waarde van het gist DFNA5-model te bepalen.

Vervolgens screenden we een collectie van achttien deletiemutanten, giststammen die een specifieke apoptotische component missen, met het oog op het identificeren van DFNA5-modulatoren. Van elke gist deletiestam werd er een groeiprofiel opgesteld, aangevuld met verschillende apoptotische kleuringen, en dit werd vervolgens vergeleken met de achtergrond stam. Deze screening resulteerde in de identificatie van verschillende mitochondriële proteïnen betrokken bij DFNA5-geïnduceerde

celdood, namelijk Fis1 en Por1, en Aac1 en Aac3, zijnde respectievelijk inhibitoren en activatoren van DFNA5-gemedieerde celdood. Aangezien deze vier proteïnen allen betrokken zijn bij mitochondriële processen was dit een eerste aanwijzing voor een mogelijke mitochondriële rol in het DFNA5-mechanisme. Bovendien, demonstreerden we dat het proteïnedegradatiemechanisme in gist ontoereikend bleek voor de afbraak van mutDFNA5 in tegenstelling tot wtDFNA5.

De rol van de mitochondriën werd verder aangetoond door een Agilent-microarraystudie uitgevoerd in gist. Transformatie van mut*DFNA5* leidde tot de opregulatie van verschillende mitochondriële processen, zoals het ATP-gekoppelde elektronentransport en het respiratieproces, en de neerregulatie van proteïnevouwing en processen gerelateerd aan het endoplasmatisch reticulum (ER). Deze resultaten bevestigen inderdaad de bovengenoemde resultaten en wijzen een belangrijke rol aan van de mitochondriën en mogelijks ook van het ER in mutDFNA5-geassocieerde celdood in *Saccharomyces cerevisiae*.

In het tweede deel van deze thesis maakten we gebruik van humane HEK293T-cellen. Een eerder uitgevoerd Illumina-microarrayexperiment werd opnieuw geëvalueerd en aangevuld met een additionele *gene ontology*-analyse voor de identificatie van DFNA5-gerelateerde pathways. Twee belangrijke processen werden hierbij geïdentificeerd. Allereerst zagen we een opregulatie van de MAPK-pathways, welke was gekenmerkt door fosforylatie van het ERK- en het JNK- proteïne. Specifieke inhibitie van het JNK-proteïne reduceerde het celdood-inducerend effect van mutDFNA5, wat aantoonde dat de MAPK-pathway betrokken is bij de inductie van het DFNA5-mechanisme. Voorts zagen we ook een neerregulatie van processen geassocieerd aan proteïnevouwing wat kan wijzen op een potentiële rol van het ER in humane HEK293T-cellen.

Om de rol van de mitochondriën verder te onderzoeken in humane cellijnen, voerden we verschillende mitochondriële assays uit. Deze assays demonstreerden een gedeeltelijke reductie van de mitochondriële membraanpotentiaal aangevuld met een verhoogde ROS-productie, vrijstelling van zowel cytochroom c en het matrix citraatsynthase. Deze mitochondriële veranderingen waren bovendien caspase-onafhankelijk.

Onderzoek van het mitochondriële netwerk, gebruik makend van fluorescentiemicroscopie, toonde bovendien desintegratie van het mitochondriële netwerk aan. Dit werd gekenmerkt door sferische aggregaten en de afwezigheid van een tubulair, intact netwerk zoals geobserveerd in de controle stalen.

Deze data demonstreren dat expressie van mut*DFNA5* in humane HEK293T-cellen resulteert in mitochondriële beschadiging gevolgd door celdood. De connectie tussen de mitochondriën, de MAPK-pathway en het ER in DFNA5-geassocieerde celdood blijft echter onduidelijk op dit moment. Verder onderzoek naar DFNA5-gemedieerde processen zal leiden tot een beter inzicht in DFNA5-geassocieerd GV en tot GV gerelateerd aan mitochondriële celdood in het algemeen.

II. Abstract

In 1997, *DFNA5* was originally identified as a gene responsible for an autosomal dominant non-syndromic form of hearing loss (HL). Today, already eight families with HL have been reported with mutations in *DFNA5*. Four of these mutations differ at genomic level, but they all result in skipping of exon 8 leading to a truncated immature protein. Skipping of exon 8 causes a frameshift of the open reading frame, leading to a shortened version of the unmutated wild-type DFNA5 protein (wtDFNA5), namely mutant DFNA5 (mutDFNA5).
In addition to HL, wt*DFNA5* has also been correlated with different forms of cancer, such as breast, colorectal, gastric and melanoma cancer. In these tumours, *DFNA5* is epigenetically inactivated by hypermethylation. Furthermore, overexpression of *DFNA5* in tumour cell lines resulted in reduced tumour growth and colony size which led to the hypothesis that *DFNA5* is a tumour suppressor gene.

Gregan *et al.* (2003) were the first to demonstrate that mutDFNA5 induced a growth defect in fission yeast, but the exact function of DFNA5 remained unknown for a long time. Recently however, functional studies on *DFNA5* identified the gene as an apoptosis-inducing gene in human cell lines. As already demonstrated by Gregan *et al.*, the DFNA5-induced cell death mechanism seems to be at least partially conserved between yeast and human cell lines. Therefore, the first part of this thesis was performed using *Saccharomyces cerevisiae* as a model organism. A *DFNA5*-humanised yeast model was developed in the BY4741 background strain, transformed with either wt*DFNA5* or mut*DFNA5*, and validated by several cell death assays to determine the value of yeast as a model organism for DFNA5.
Next, an apoptosis deletion strain collection, a collection of eighteen yeast strains deleted in one particular gene related to apoptosis, was screened to identify modulators of the DFNA5 cell death mechanisms in yeast. Growth profiles of every yeast strain and several apoptotic stainings were performed and compared to the background strain. This identified several mitochondrial proteins related to DFNA5-induced cell death. The Fis1, Por1,

and, Aac1 and Aac3 proteins were characterised as respectively inhibitors and inducers of DFNA5-related cell death. These proteins were related to either the mitochondrial dynamics or the permeabilisation process, revealing for the first time an important role for the mitochondria.
Additionally, wtDFNA5 was subject to normal protein degradation in yeast, whereas mutDFNA5 seemed to escape this protein quality control. As the endoplasmic reticulum (ER) is involved in protein folding and degradation, these results could suggest an additional role for the ER. As both organelles, the mitochondria and the ER, are tightly correlated, interplay between the ER and the mitochondria could be important for DFNA5-induced cell death in yeast.
Later on, an Agilent microarray study performed in yeast confirmed the role of the mitochondria as several gene ontology mitochondria-related processes, such as ATP-coupled electron transport and respiration, were up-regulated upon mut*DFNA5* transformation in yeast. Additionally, processes related to protein folding and the endoplasmic reticulum (ER) were down-regulated upon mut*DFNA5* transformation. These data validated the former results indicating a role for the mitochondria and a potential role for the ER.

The second part of this thesis was performed in human HEK293T cells. A former Illumina microarray experiment was re-evaluated by an additional analysis investigating the gene ontology-enriched annotations. This microarray experiment identified two main features, namely the up-regulation of the MAPK pathways and the down-regulation of the protein folding process upon mut*DFNA5* transfection. The up-regulation of the MAPK pathway was characterised by phosphorylation of JNK and ERK. Specific inhibition of JNK partially abolished the mutDFNA5-induced cell death, indicating the importance of this pathway in the DFNA5-associated cell death mechanism. Down-regulation of gene ontology processes related to protein folding again revealed a potential role for the ER in DFNA5-associated mechanisms.

Because the prior research in yeast identified several mitochondrial proteins, several mitochondrial aspects were additionally investigated in

human HEK293T cells. This revealed partial loss of the mitochondrial membrane potential, increased ROS production, release of both cytochrome c and the matrix citrate synthase. These events were independent on caspases. The mitochondrial network was further investigated by fluorescence microscopy and revealed the accumulation of damaged mitochondria in mut*DFNA5*-transfected HEK293T cells. This was demonstrated by the presence of spherical aggregates and the absence of a tubular integer mitochondrial structure which was observed in the control samples. These results indicate the presence of dysfunctional mitochondria upon expression of mutDFNA5 leading to mitochondrial collapse and eventually cell death. The link between the mitochondria, the MAPK pathway and the ER in DFNA5-related cell death, however, remains unknown at this moment. Additional research is needed to further investigate DFNA5-related processes, which will lead to new insights for DFNA5-associated HL and, more in general, for HL related to mitochondrial cell death.

III. Abbreviations

ΔΨ	Mitochondrial membrane potential
3-MA	3-methyladenine
Aac1/3	ADT/ATP carriers
Adh2	Alcohol dehydrogenase
AFG3L1	ATPase family gene-3
AIF	Apoptosis-inducing factor
ANT	Adenine nucleotide translocase
Apaf-1	Apoptosis protease-activating factor 1
ARHI	Age-related hearing impairment
ATG	Autophagy-related genes
ATP	Adenosine triphosphate
AV	AnnexinV
BCL2	B-cell lympfoma 2
Beclin	BCL2-interacting protein
BH	BCL2 homology
BIR	Baculovirus IAP repeats
Caf4	CCR4-associated factor
CARD	Caspase recruitment domain
CDC19	Pyruvate kinase CDC19
CF	Cytosolic fraction
COX	Cytochrome c oxidase
CsA	Cyclosporine A
Cy3/5	Cyanine 3/5-CTP
CypD	Cyclophilin-D
DAPI	4',6-diamidino-2-phenylindole
DDP	Diadenosine and Diphosphoinositol Polyphosphate phosphohydrolase
DFNA5	Deafness, autosomal dominant 5
DHE	Dihydroethidium
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified eagle's medium
DNAJB 1	DnaJ (Hsp40) homolog, subfamily B, member 1
Dnm1p	Dynamin-related
DRP1	Dynamin-related protein
EGFP	Enhanced green fluorescent protein

EGR1	Early growth response 1
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERK	Extracellular signal-related kinase
FADD	Fas-associated death domain
FITC	Fluorescein isothiocyanate
FM-4-64	N-(3-triethylammoniumpropyl)-4-(p-diethylamino phenylhexa-trienyl) pyridinium dibromide
Fzo1	Fuzzy onion 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDAP	Ganglioside-induced differentiation-associated protein 1
GO	Gene ontology
GSDM	Gasdermin
H_2O_2	Hydrogen peroxide
HA	Hemaglutinin
HCA	Hydrophobic cluster analysis
HEK293T	Human embryonic kidney
hFIS/Fis1	(Human) fission protein
HK	Hexokinase
HL	Hearing loss
HMBG 1	High mobility group Box 1 protein
HRP	Horse radish peroxidase
Hsp90	Heat shock protein 90
HSPA6	Heat shock 70kDa protein 6
HTRA2	High temperature requirement serine protease
IAP	Inhibitor of apoptosis
IMM	Inner mitochondrial membrane
IPOD	Insoluble protein deposit
JNK	c-Jun N-terminal kinase
JUNQ	Juxtanuclear quality control
kD	Kilo Dalton
LC3	Microtubule-associated process 1 light chain 3
MAPK	Mitogen-activated protein kinase
Mdm30/36	Mitochondrial distribution and morphology
Mdv1	Mitochondrial division
ME	Mid-exponential

Mca	Metacaspase (yeast caspase)
MF	Mitochondrial fraction
MFF	Mitochondrial fission factor
MFN1/2	Mitofusin 1and 2
Mgm1	Mitochondrial genome maintenance
Mgr2	Mitochondrial genome required 2
MIB	Mitofusin-binding protein
MIEF/Mid49/51	Mitochondrial elongation factor 1
mitoPLD	Mitochondria-associated phospholipase D
MLKL	Mixed lineage kinase domain-like
MOMP	Mitochondrial outer membrane permeabilisation
MPT	Mitochondrial permeability transition
mPTP	Mammalian mitochondrial transition pore
MSBR3	Methionine sulfoxide reductase B3
MTGM	Mitochondrial targeting GxxxG motif protein
MTP18	Mitochondrial protein
MTS	Mitochondrial targeting signal
mutDFNA5	Mutant DFNA5
NIHL	Noise-induced hearing loss
Nma111	Nuclear mediator of apoptosis
Nuc1	Endonuclease G
Num1p	Nuclear migration protein
OD	Optical density
OMA1/MPRP-1,	Metalloprotease-related protein-1
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
PARL	Presenilin-associated rhomboid-like
PCD	Programmed cell death
Pcp1p	Processing of cytochrome c peroxidase
PD	Post-diauxic
PGK1	Phosphoglycerate kinase
PI	Propidium iodide
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
Por	Porin
PPP	Pentose phosphate pathway
PTPC	Permeability transition pore complex
RIPK1/3	Receptor-Interacting serine/threonine protein kinases

ROS	Reactive oxygen species
rtPCR	Reverse transcriptase PCR
S. cerevisiae	*Saccharomyces cerevisiae*
siRNA	Short interfering RNA
SMAC	Second mitochondria-derived activator of caspase
t-BHP	Tert-butylhydroperoxide
TDH2/3	Triose-phosphate Dehydrogenase
Timm8a	Translocase of the inner mitochondrial membrane
TM7SF2	Transmembrane 7 superfamily member 2
TMRM	Tetramethyl rhodamine methylester
TNF	Tumour necrosis factor
TOR	Target of rapamycin
TPI	Triose phosphate isomerase
TRAF2	TNF receptor-associated factor 2
TSG	Tumour suppressor gene
UCP 2	Uncoupling protein 2
Ugo1	Means fusion in Japanese
UPR	Unfolded protein response
Ups1	Unprocessed
VDAC	Voltage-dependent anion channel
VPS33B	Vacuolar protein-sorting 33 homolog B (yeast)
wtDFNA5	Wild-type DFNA5
Ybh3p	Yeast BH3-only protein
Yca/Mca	Yeast caspase/Metacaspase
YME1L	Yeast mitochondrial escape
yPTP	Yeast mitochondrial transition pore
Ysp2	Yeast suicide protein
zVAD-fmk	N-benzyl-oxycarbonyl-Val-Ala-Asp -fluoromethylketone

Part I

Introduction

Chapter I

Introduction

1 Introduction

DFNA5 was originally identified as a gene responsible for a dominant autosomal non-syndromic form of hearing loss (HL) in a Dutch family [1]. Today, already eight families have been identified with mutations in *DFNA5* associated with HL [2-7]. HL is a very diverse and complex disorder reflected by the many different genes that have been identified [8, 9]. The past decades, genetic research on HL has identified many different loci and causative genes. Today, more than 30 genes responsible for an autosomal dominant non-syndromic form of HL have been identified. Additionally, 55 autosomal recessive non-syndromic genes and 4 non-syndromic X-linked genes were found to be associated with hereditary hearing problems (http://hereditaryhearingloss.org). It is estimated that around 1 in 1000 children are born deaf or will develop hearing problems in early childhood and most of the time this is attributable to genetic factors [10-12]. In addition, about half of the people over age 75 will develop severe hearing problems during aging, which makes it one of the most common sensory deficits in the elderly [13]. Furthermore, in the last decade, an increase of the prevalence of HL among young adults has been recorded due to excessive use of music devices [14-16]. HL is hence a very common and underestimated health problem which leads to social isolation, stigmatisation and reduced quality of life.

The function of *DFNA5* remained unknown for a long time, but recently DFNA5 has been associated with apoptosis, a specific form of programmed cell death (PCD) [17]. Apoptosis is a very complex and highly regulated mechanism. It is one of the major routes of cell death associated with numerous physiological processes and major implications upon deregulation. It is therefore essential to maintain the apoptotic process in balance and disturbance of this delicate task leads to a variety of pathologies, such as cancer and neurodegeneration but also HL [18-20].

The role of apoptosis in the pathology of HL has been well studied and plays a prominent role, especially in the development of the vertebrate inner ear

and in the morphogenesis of the semicircular canals [21]. Due to its prominent role in hearing impairment, several types of HL, such as age-related hearing impairment (ARHI), noise-induced hearing loss (NIHL) and monogenic forms of HL, like *DFNA5*-induced HL, are linked to apoptosis. Genetic research on HL has identified many different genes and some of them were involved in apoptotic pathways, such as *DFNA5*, *TJP2* and *Smac/DIABLO* [16, 22, 23]. Additionally, a clear link between ARHI and apoptosis has been established as demonstrated by several mouse models used to study ARHI. Commonly used mouse strains for ARHI are CBA/J and C57BL/6, which both showed increased oxidative stress and enhanced apoptotic activation in the aged cochleas of these mouse models [24, 25]. Finally, apoptotic damage was reported in several animal models following acoustic overstimulation demonstrating a link between NIHL and apoptosis [26, 27].

As *DFNA5* was recently identified as a gene related to apoptosis [17] this introduction will highlight some important aspects of apoptosis and other forms of PCD. The emphasis will lie on the mitochondria as these are key players in the regulation of PCD. Mitochondria serve as docking sites for several PCD proteins and play a decisive role for either survival or cell death. After a short summary about the current knowledge of DFNA5, two specific aspects of the mitochondria, namely their dynamics and the mitochondrial permeabilisation process, will be discussed.

1.1 DFNA5

1.1.1 DFNA5 and hearing loss

In 1966, Huizing *et al.* described for the first time a Dutch family with severe hearing problems [28]. Until 1985, this family was followed up and genetic research in 1995 localised the candidate gene to chromosome 7p15 [29]. Further refinement of this region led to the identification of a complex mutation in a gene expressed in the cochlea which co-segregated with HL in this family. The gene was termed *DFNA5* as no function could be assigned [16]. The HL was inherited in an autosomal dominant way with a penetrance of 100% and no other phenotypic abnormalities were seen, classifying the HL as non-syndromic.

Nowadays, eight families have already been identified with mutations in *DFNA5*-associated with HL. Comparison of a Korean, a Chinese and two Japanese *DFNA5* families resulted in the identification of a putative founder mutation in East-Asians [2, 6]. Four of these eight mutations differ at the genomic level but they all result in the skipping of exon 8 leading to an immature truncated protein (Table 1) [3-6].

The phenotype of *DFNA5*-related HL is very similar in all these families with the exception of the age-of-onset (Table 1). HL related to *DFNA5* is symmetric, meaning that both the right and the left ear are similarly affected. The age-of-onset of *DFNA5*-related HL varies between 15 year and 50 year and no vestibular problems are observed. The HL starts at the higher frequencies but eventually spreads to the lower frequencies. Due to the relative slow speech recognition deterioration, it is assumed that DFNA5-related HL is associated to cochlear dysfunction and is not an auditory neuropathy.

Table 1 Summary of reported DFNA5 mutations and clinical features

Family origin	Mutation	Location	Mutation effect	Age-of-onset	HL progressive	Audiograms	Vestibular symptom	References
Dutch	c.990+503_990+1691 del ins132	Intron 7	Skipping of exon 8	5-15	+	HF	-	Van Laer et al. 1998
Chinese	c.991-15_991-13del	Intron 7	Skipping of exon 8	7-30	+	HF	N.R.	Yu et al. 2003
Dutch	c.991-6C > G	Intron 7	Skipping of exon 8	0-40	+	HF	-	Bischoff et al. 2004
Chinese	c.1183+4A > G	Intron 8	Skipping of exon 8	11-50	+	HF	-	Cheng et al. 2007
Korean	c.991-15_991-13del	Intron 7	Skipping of exon 8	10-19	+	HF	-	Park et al. 2010
Chinese	c.991-2A > G	Intron 7	Skipping of exon 8	8-18	+	HF	N.R.	Chai et al. 2014
Japanese	c.991-15_991-13del	Intron 7	Skipping of exon 8	10-30	+	HF	-	Nishio et al. 2014
Japanese	c.991-15_991-13del	Intron 7	Skipping of exon 8	18	+	HF	-	Nishio et al. 2014

HL, Hearing loss; HF, high frequency; N.R., not reported

1.1.2 DFNA5 and cancer

Additionally to hearing impairment, *DFNA5* has also been linked to several forms of cancer. The first indication that *DFNA5* was not only related to HL but also to cancer was established in 1998 by Thompson *et al.* [30]. This study searched for differentially expressed genes between hormone-responsive and hormone-unresponsive breast carcinomas. Hormone-responsive breast cancer is associated with the expression of the oestrogen receptor and is clinically less aggressive compared to oestrogen receptor negative tumours. Thompson *et al.* identified an inverse correlation between the expression of *DFNA5* and oestrogen receptor. Since then, *DFNA5* has been associated with breast, colorectal, melanoma, gastric and hepatocellular cancer [30-35].

In several different forms of cancer, such as breast, colorectal and gastric cancer, wild-type *DFNA5* (wt*DFNA5*) is epigenetically inactivated by methylation resulting in a reduced amount of *DFNA5* expression. Because the percentages of *DFNA5* methylation in these tumour cells are very high, ranging from 52% to 65% in respectively gastric and colorectal tumour tissue, it was hypothesised that *DFNA5* is a tumour suppressor gene (TSG). Enhancement of full-length *DFNA5* expression decreased cell growth and resulted in a reduction in colony number. Whereas *DFNA5* down-regulation increased cell growth and colony size of the cancer cell lines, which further confirmed this hypothesis [31-33].

Additionally, *DFNA5* expression is also regulated by p53, one of the most frequent genetic altered TSG [36-38]. A screen for p53-regulated genes in responses to cellular stress revealed the transcriptional activation of *DFNA5* by a p53-responsive sequence in the gene. Furthermore, a p53-mediated apoptotic response was enhanced by *DFNA5* expression [36].

The physiological relevance of *DFNA5* in cancer is unclear at this moment, but a link of DFNA5 and apoptosis, a mechanism frequently disturbed in several forms of cancer, has been demonstrated several times. Enhanced caspase-3-mediated apoptosis was seen in *DFNA5*-transfected melanoma cell lines, which was the first time that DFNA5 was associated with apoptosis. Furthermore, expression of apoptosis-related proteins, such as

caspase-8 and Fas, was also significantly increased in hepatocellular carcinoma upon *DFNA5* expression [34, 35]. Finally, *DFNA5* expression has been associated with lymph node metastasis in breast cancer [32]. The high methylation percentages and the correlation with cellular invasiveness support the *DFNA5* properties as a TSG and underlie the potential of *DFNA5* as a biomarker for certain forms of cancer.

1.1.3 Functional studies on DFNA5

The function of DFNA5 remained unknown for a long time due to the fact that *DFNA5* has no clear structural or functional similarities with other genes. Gregan *et al.* (2003) were the first who demonstrated a toxic effect of mutant *DFNA5* (mut*DFNA5*) upon transformation in fission yeast [39]. They also identified a conserved zinc-finger-like motif, a CCCH domain, which has sequence similarity with the fusion yeast gene *Mcm10*, a gene related to DNA replication. It is however, very unlikely that they share functional similarity because *DFNA*5 was not able to complement the fission yeast *Mcm10* homologue, the cdc23 mutant [39].
Besides *Mcm10*, one other closely related gene to *DFNA5* was identified through phylogenetic analysis, namely *Pejvakin* [40]. *Pejvakin* is associated with an autosomal recessive non-syndromic form of HL and shares a 250 amino acid N-terminal stretch with *DFNA5* revealing a common ancestor [41, 42]. *DFNA*5 and *Pejvakin* both belong to the gasdermin family, a group of genes with a high tissue-specific expression pattern in the gastrointestinal tract. They all share this N-terminal conserved region. Despite this, *DFNA5* and *Pejvakin* are phylogenetically distant from other gasdermin members and form a distinct clade in this family. Additionally, due to the gastrointestinal specific expression profile of the gasdermin family, it is very unlikely that they have similar molecular functions. Until today, the current function of Pejvakin remains unknown revealing no further information on DFNA5, until a few years ago.

A few years ago, functional studies on DFNA5 in human cell lines revealed a direct link with apoptosis [17]. Transfection of mut*DFNA5* in human HEK293T cells resulted in enhanced apoptotic cell death. This was demonstrated by the externalisation of phosphatidylserines and the

presence of DNA fragmentation, two hallmarks of apoptosis. This was the first indication about the function DFNA5 and the starting point for this thesis.

The same functional study also gave a first indication about the structure of DFNA5. This was established by a hydrophobic cluster analysis (HCA), a structural prediction analysis based on the hydrophobicity of the amino acid sequence. The HCA analysis revealed that DFNA5 contains two globular domains (exon 2-6 and exon 7-10) connected by a hinge region (exon 6-7) (Fig. 1).

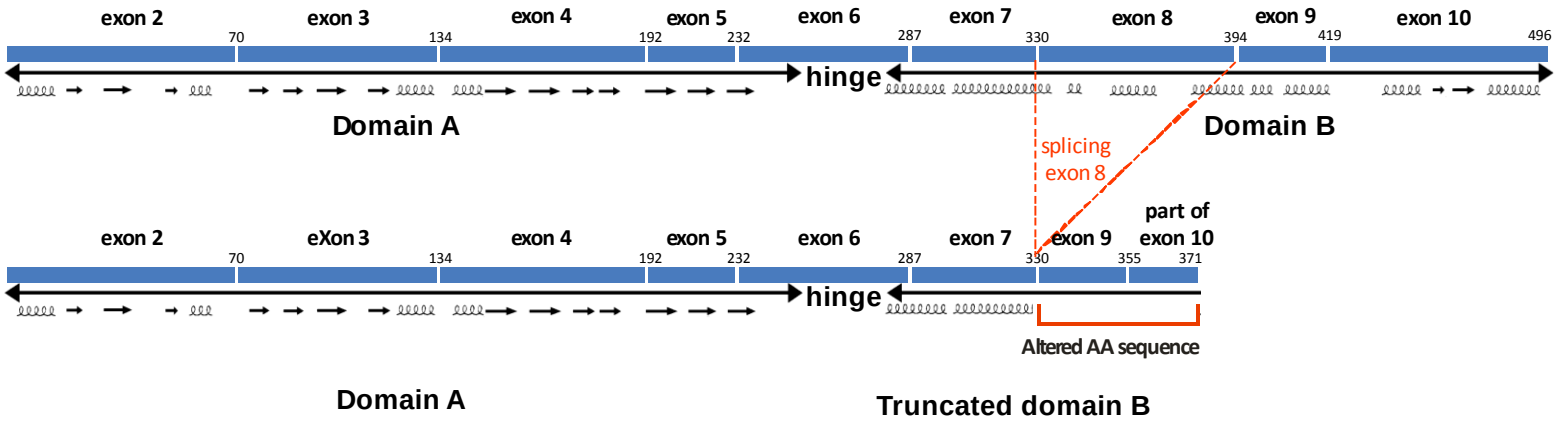


Fig. 1: DFNA5 protein structure aligned with the coding exons. The upper part shows the structure of the wtDFNA5 protein aligned with the coding exons in relation to the two globular domains, domain A and B, separated by a hinge region, as defined by the HCA analysis [17]. The lower part demonstrates the splicing mutation of the wtDFNA5 protein, namely mutDFNA5. Skipping of exon 8 results in an ORF shift. This leads to a truncated protein due to a premature stop codon in exon 10, lacking a part of domain B. The predicted β-sheets and α-helices are shown by respectively arrows and helices. AA, amino acid; ORF, open reading frame.

This structure is shared by all gasdermin members, although the length of the hinge region and the form of the second globular domain can differ. The mutations in *DFNA5* described in the different HL families, led to exon 8 skipping resulting in an immature, truncated protein lacking the second globular part of this protein (Fig. 1 and 2). Furthermore, it was demonstrated that the first part of this protein, exon 2-6, was the apoptosis-inducing part, the part shared between wt*DFNA5* and mut*DFNA5*. This observation led to the hypothesis that the second, non-apoptotic part of DFNA5 shields the first, apoptotic, part inhibiting its activation (Fig. 2, [17]).

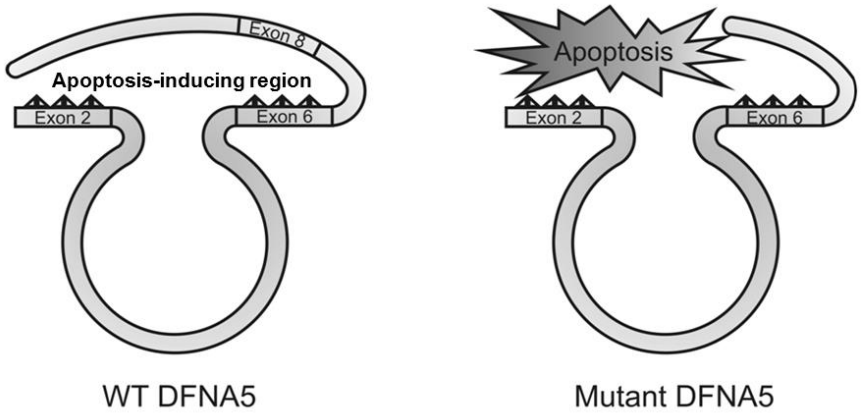


Fig. 2: Hypothetical model of the structure of DFNA5 showing wtDFNA5 and mutDFNA5. The genomic mutations, present in the different families with HL, result in the skipping of exon 8 leading to an immature truncated protein. It was demonstrated that the first part (exon 2-6) is the apoptosis-inducing part. It is hypothesised that the second part shields the first activating part and that due to the partial lack of this second part (exon 7-10), mutDFNA5 is constitutively active.

Despite our limited knowledge about the function of *DFNA5*, the association with apoptotic cell death and the structural elucidations enabled us to make an assumption on the link between *DFNA5*-mediated HL and cancer. In HL, genomic mutations in *DFNA5* result in a truncated protein lacking the second globular part. We hypothesised that this prevents the shielding of the first apoptotic region leading to a constitutively activated protein. Over activation, meaning excessive apoptotic cell death, will probably cause degeneration of the inner hair cells and eventually lead to HL. In cancer,

hypermethylation of *DFNA5* results in decreased activation of *DFNA5* and inadequate cell death, probably enabling tumour formation. This again demonstrates the importance to maintain the delicate apoptotic balance, essential for cellular homeostasis.

1.1.4 Yeast as a model organism

As already shown by Gregan *et al.* (2003), transformation of mut*DFNA5* in fission yeast induced a growth defect suggesting that the DFNA5-induced cell death mechanism seems to be at least partially conserved in yeast [39]. Therefore, a yeast model organism was used to further investigate the DFNA5-related mechanisms. The use of yeast as a model organism has several advantages, but the question remains whether yeast could be used as a model organism for a gene related to PCD.

1.1.4.1 Advantages of yeast as a model organism

Since the elucidation of the yeast genome in 1996, this model has been a pioneer organism in functional genomics and system biology for several reasons [43]. Much of the newest cellular techniques, such as high throughput arrays, are often pioneered and validated in yeast. The genetic tractability and the existence of a genome wide collection of deletion strains make yeast a very attractive model organism [44, 45]. Since the unravelling of the yeast genome, a deletion collection of every non-essential gene has been produced. This created a lot of opportunities where yeast can be used to study protein and/or gene interactions on high throughput level [46].
In addition to the high throughput applications and the genetic tractability, the high degree of conservation between yeast and human makes the model organism very valuable. Up to 30% of known genes involved in human diseases have an orthologue in yeast and nearly 1000 yeast genes (~17% of the yeast genome which has 6000 genes) are orthologues of gene families correlated with human diseases [47]. Comparison of the human genome with the yeast genome does not always reveal this homology, but yeast can often give a first clue about the functional annotation of a gene. The speed of growth, the price and the relatively easy control of cell division and cell growth are additional reasons making yeast a valuable tool to the knowledge of the eukaryote biology.

1.1.4.2 Physiological role of programmed cell death in yeast

It has been a debate for a long time whether PCD does exist in yeast, a unicellular organism. What would be the purpose and the benefit for a unicellular organism to have a suicide program? Since the publication of the yeast genome, numerous studies were performed on this topic and revealed that yeast cannot solely be seen as a unicellular organism being an isolated cell without any communication. In fact, yeast behaves like a multicellular community where cell death can be beneficial for the whole colony. In this point of view, cell death can be seen as an altruistic mechanism to serve the fitness of the population [48]. There are several reasons for yeast to commit suicide. During chronological aging of a yeast population, the older, more damaged cells die by PCD [49]. The older yeast cells thereby release nutrients which can be reused by the younger and fitter cells. In addition, they also secrete certain pro-survival factors stimulating the proliferation of the colony. During mating, yeast cells also act like a community where PCD serves the benefit of the colony. In the absence of a suitable mating partner, yeast cells die, eliminating damaged or infertile cells. This promotes a diploid state which enhances genetic diversity and adaptive regrowth capacity, ensuring overall survival of the population [48, 50].

Some yeast strains, known as virus-encoded killer yeast, produce certain toxins encoded by cytosolic double-stranded RNA viruses. Killer yeast themselves are immune for those toxins but they can induce apoptosis in susceptible yeast strains under competing circumstances such as nutrient depletion. Here, apoptosis is misused by some yeast strains which use this mechanism as a competitive benefit for survival [51, 52].

1.1.5 Conclusion

DFNA5 is a gene associated with a dominant autosomal non-syndromic form of HL and is correlated with several forms of cancer. In human cell lines, *DFNA5* was shown to induce cell death related to apoptosis. Additionally, DFNA5 was also shown to induce a growth defect in a fission yeast model, suggesting that the DFNA5 PCD mechanism is at least partially conserved between yeast and metazoan. As PCD was the main research field throughout this thesis, the next part of the introduction will highlight some

important aspects about PCD in general, and on mitochondria in particular. As yeast was initially used as a model organism to study the DFNA5-related mechanisms, a comparison will be made between the yeast and mammalian apoptotic pathway discussing the resemblances and the differences.

1.2 Programmed cell death

Cell death is a fundamental aspect of life and is closely related to health and disease. Many different forms of cell death have been identified over the past few decades. Therefore, cell death is a very complex and highly regulated cellular mechanism important during development, organogenesis, the function of the immune system and tissue homeostasis [21, 82] . Many different types of cell death co-exist and an orchestrated interplay between them is highly important. Deregulation has many implications in different pathologies, such as auto-immune diseases [18] and cancer [19, 53]. Autophagy, apoptosis and necrosis are the main cell death pathways in mammalian cell lines. For a long time, only apoptosis and autophagy were considered as programmed forms of cell death contrasting necrosis which was regarded as an accidental process. However, research of the last few decades challenged this idea by the identification of several regulators of necrosis, creating a new type of cell death, namely regulated necrosis or necroptosis.

1.2.1 Introduction to programmed cell death: Autophagy and Necrosis

Initially, autophagy was classified to have a pro-survival and protective role in cells. Its main function is to remove damaged organelles during starvation by a self-digestion process providing metabolites which can be reused. Formation of autophagosomes, double membrane vesicles, is a key step in this process which is essential for sequestering of components to be degraded and for delivering them to lysosomes [54, 55]. Different steps are involved in the autophagic process starting from initiation, recognition and engulfment of components to be degraded, nucleation, expansion and eventually fusion of the vesicle with the lysosome [55-57]. Already 31 autophagy-related genes (*ATG*) have been identified which can be classified into groups depending on their functional role in one of the autophagic steps. Most of these *ATG* genes have been identified in yeast and they have

similar roles in higher eukaryotes. TORC1 or the RAS/cAMP PKA pathway for example, regulate autophagy both in yeast and in mammals [58-62]. Despite its role in survival, autophagy is also correlated with cell death and implicated in a wide range of diseases [56, 63, 64]. The close relation between autophagy and different forms of cell death, such as apoptosis and necrosis, has been well studied. A variety of stimuli can activate different forms of cell death, including autophagy. Depending on the trigger or cell type, autophagy can either stimulate or block cell death [65].

For a long time, necrosis was regarded as a passive, unregulated form of cell death. Necrosis can be distinguished from other forms of cell death by the presence of swollen organelles, rupture of the plasma membrane, no caspase activation and the largely intact nucleus. Necrosis is often related to inflammatory responses triggered by spoilage of cytoplasmic content due to rupture of the plasma membrane [66, 67]. Later on, it became clear that necrosis is more than just an accidental form of cell death and some even claim that all forms of necrosis should be considered as potentially regulated [68, 69]. This consideration would have major implications at the therapeutic level which should be carefully investigated. Programmed necrosis or necroptosis can be triggered either by activating the death receptors present at the plasma membrane, similar to apoptosis, or by pathogen recognition receptors present at cells of the innate immune system [70, 71]. The main regulators of necroptosis are the receptor interacting serine/threonine protein kinases (RIPK1/3) and mixed lineage kinase domain-like (MLKL) protein [72, 73].

Similar to metazoan, evidence of necrotic cell death under normal physiological circumstances in yeast is mounting, suggesting the existence of regulated necrosis. One of the most prominent examples of necrosis under normal conditions is the exhibition of morphological necrotic features during chronological aging while lacking apoptotic markers. Nuclear release of Nhp6Ap, the HMGB1 (high mobility group Box 1 protein) yeast homologue and the marker for necrotic cell death, was demonstrated in aged yeast cells [74, 75]. Furthermore, different organelles and genetic

factors have been identified in yeast to mediate necrosis. The yeast Hsp90p for example, is one of the factors which have been associated with necrotic regulation harbouring a pro-death role [76, 77]. Additionally, the vacuoles are closely connected to necrotic cell death being responsible for maintaining the intracellular pH. Dysfunctional vacuoles induce necrosis due to a collapse in intracellular pH hereby releasing pro-necrotic proteases resembling the induction of necrotic cell death by high concentrations of acetic acid [78, 79].

1.2.2 Apoptosis, a specialised form of PCD

1.2.2.1 Metazoan

Apoptosis is a complex and highly regulated mechanism. Typical apoptotic hallmarks are nuclear condensation and fragmentation, DNA cleavage, externalisation of phosphatidylserine providing an 'eat-me signal' and formation of apoptotic bodies. Apoptosis is an active, energy-consuming suicidal process contrasting necrosis and necroptosis which will occur under low energy conditions [80, 81].

Two major apoptotic pathways are present in mammalian cell lines, the extrinsic or death receptor-mediated pathway and the intrinsic or mitochondria-related pathway [82]. Despite different activation triggers, a similar pattern is followed once activated. Triggering one of the cell death pathways activates different initiator caspases by cleavage followed by cleavage of effector caspases [83, 84] (Fig. 3).

The receptor-mediated pathway is activated by extracellular binding of different ligands, such as FasL, TNF and TRAIL, to their transmembrane death receptors [85-87]. Upon binding to its receptors, a Death-Inducing Signalling Complex (DISC) is formed by recruitment of the adaptor death domain (FADD or TRADD dependent on the ligand) and procaspase-8 [86, 88, 89]. Proteolytic activation of procaspase-8 initiates the caspase cascade leading to apoptosis [90] (Fig. 3).

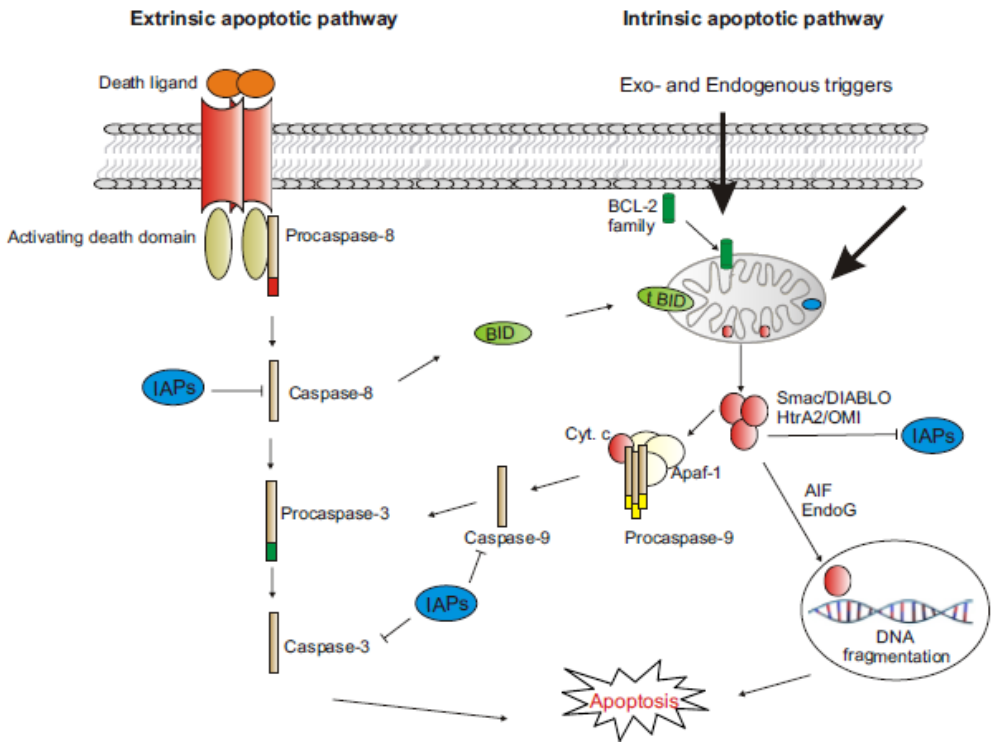


Fig. 3: The apoptotic pathway in metazoan. The extrinsic pathway is activated by binding of different death ligands on their receptor. An activating death domain is recruited, which in turn activates the initiator procaspase-8 by cleavage. The intrinsic pathway is induced by either exo- or endogenous triggers. The mitochondria are the key players in this pathway which are regulated by different pro- and anti- apoptotic BCL2 proteins (dark green). Pro-apoptotic BCL2 proteins translocate to the mitochondria upon apoptosis induction and mediate mitochondrial permeabilisation. This results in the release of several apoptotic factors indicated by red circles. The initiator procaspase-9 is activated by the release of cytochrome c. Both the extrinsic and intrinsic pathway will result in activation of the effector procaspase-3 where both pathways converge. Cleavage of BID by caspase-8 demonstrates the interplay between the two pathways [91, 92]. Other released factors are AIF and EndoG, which are translocated to the nucleus to cleave DNA, and Smac/DIABLO and HtrA2/Omi which can mediate apoptosis by inhibiting IAP proteins. IAP, inhibitor of apoptosis; tBID, truncated BID; AIF, apoptosis-inducing factor; EndoG, endonucleaseG; Apaf-1, apoptosis protease-activating factor; SMAC, second mitochondria-derived activator of caspase; DIABLO, direct IAP-binding protein with low pI; Cyt.c, cytochrome c; HtrA2, high temperature requirement serine protease; BCL2-2; B-cell lympfoma 2.

The intrinsic apoptotic pathway is mediated by the mitochondria. Mitochondria are activated by different stimuli, both extra- and intracellular, such as DNA damage, oxidative stress or radiation [93, 94]. Triggering the mitochondrial pathway releases several pro-apoptotic proteins, such as cytochrome c, Smac/DIABLO (second mitochondria-derived activator of

caspase/direct IAP-binding protein with low pI) and HtrA2/Omi (high temperature requirement serine protease), thereby activating the caspase cascade [92, 95, 96]. Once released from the mitochondria, cytochrome c will initiate the formation of a protein complex, named the apoptosome [97, 98]. This complex is formed by the recruitment of several Apaf-1 proteins (apoptosis protease-activating factor) and procaspase-9 (Fig.3). Monomeric Apaf-1 contains an N-terminal caspase recruitment domain (CARD) which will be displaced by binding to cytochrome c. Following cytochrome c binding, monomeric Apaf-1 will assemble with six others Apaf-1 to generate a heptametrical wheel-shaped structure which will provide a platform to increase the local concentration of procaspase-9. Through this displacement, the CARD domain is now freely available to recruit several inactive procaspase-9 proteins [99-101]. By recruiting several pro-caspases, different catalytic domains are brought in close proximity inducing dimerisation and hence activation of the caspases [102-104]. Procaspase-9 has a long prodomain which facilitates the selective recruitment by the Apaf-1 adaptor molecules [105]. Once recruited, activation of procaspase-9 will initiate the caspase cascade. Caspase-9 will cleave other effector caspases as long as it is bound to the apoptosome. Due to the length of the activation loop of caspase-9, the region between the large and the small subunits is much longer compared to other caspases, autocatalytic cleavage is not necessary. This linker is free to move and can access the active site of the effector caspases without cleavage. Autocatalytic cleavage is not essential for their function and would release caspase-9 from the apoptosome, and thereby its capacity to activate procaspase-3 [103, 106-108]. Due to the autocatalytic cleavage within the apoptosome, the initial intracellular concentration of procaspase-9 and the rate of this dissociation event will eventually determine the duration of the apoptosome activity [107, 109]. This branch of the mitochondria-related pathway is called the caspase-dependent pathway (Fig. 3).

The caspase-independent pathway is mediated by either EndoG (endonuclease G) or AIF (apoptosis-inducing factor), two factors which are

also released from the mitochondria upon apoptotic stimuli [110-112]. Once released from the mitochondria, they are translocated to the nucleus which results in DNA fragmentation, one of the typical apoptotic hallmarks (Fig. 3).
This intrinsic apoptotic pathway can be regulated by two major proteins families: the BCL2 (B-cell lympfoma) and the IAP (inhibitor of apoptosis) family. The IAPs inhibit the caspase-dependent pathway and contain one to three BIR domains (baculovirus IAP repeat) by which they bind and inhibit the caspases [113, 114]. Eight distinct IAPs have been characterised in metazoan and most of them can inhibit the proteolytic activity of caspases either directly or indirectly by ubiquitination. Some of them reside in the cytosol, nucleus or are released from the mitochondria to the cytosol upon apoptotic induction [115-117]. Upon induction of apoptosis, the IAPs can be inhibited in turn by Smac/DIABLO or Omi/HtrA2, which are both released by the mitochondria together with cytochrome c [118-121] (Fig. 3).
The other major group of proteins mediating the intrinsic pathway is the BCL2 family which can be divided in three main groups: the pro-apoptotic proteins, the anti-apoptotic proteins and the pro-apoptotic mediators, also called the BH3-only proteins [122, 123]. They all share up to four BCL2 homology (BH) domains of which the BH3-domain is the most crucial one [123]. The final stoichiometry of the pro- and anti-BCL2 proteins will eventually determine whether the apoptotic pathway will be activated [124]. Once the delicate balance is shifted towards apoptosis, some of the BCL2 proteins will translocate to the mitochondria and induce the release of several apoptotic factors (Fig. 3).

1.2.2.2 Yeast

Similar to metazoan, different forms of PCD exist in unicellular organisms, although often in a simplified version. In yeast for example, only the intrinsic apoptotic pathway is present contrasting the higher eukaryotes where both an extrinsic and an intrinsic pathway exist (Fig. 4).

Comparison of the intrinsic, mitochondrial apoptotic pathways of yeast and metazoan reveals a high degree of similarity. Besides the presence of the typical apoptotic hallmarks, such as phosphatidylserine externalisation, DNA fragmentation, ROS production and chromatin condensation, several important apoptotic regulators have been identified [125, 126]. The identification of yeast homologues of the apoptosis-inducing factor (AIF/Aif1) [127], the endonuclease G (EndoG/Nuc1) [128], HTRA2-like serine protease (HtrA2 or OMI/Nma111) [129, 130] and cytochrome c [131, 132], further demonstrates the resemblances between yeast and higher eukaryotes (Fig. 4). These factors are, with the exception of Nma111, released from the mitochondria to the cytosol upon induction of apoptosis and play a role in cell death in yeast. When released from the mitochondria, Nuc1 and Aif1 are, similar to metazoan, translocated to the nucleus where they function as nuclear mediators of cell death and cleave DNA, resembling the caspase-independent pathway [127-130].

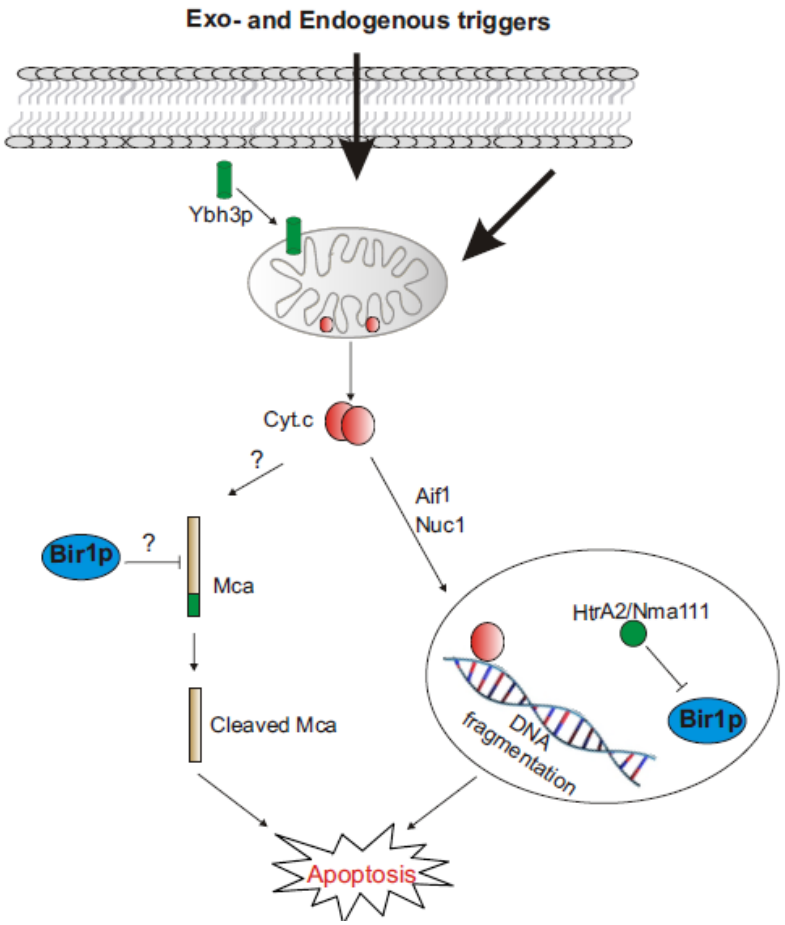


Fig. 4: Yeast apoptotic pathway. Release of several apoptotic factors, indicated by red circles, can be mediated by either exo- or endogenous triggers. Pro-apoptotic Ybh3p translocates to the mitochondria upon apoptosis induction. Release of Aif1 and Nuc1 induces DNA fragmentation preceded by their nuclear translocation. Meanwhile, the yeast caspase Yca1 (Mca) is activated by cleavage. Bir1p belongs to the IAP family and can reduce apoptosis by Mca inhibition or can be inhibited by HtrA2/Nma111. Aif1, apoptosis-inducing factor; Nuc1, endonucleaseG; Cyt.c, cytochrome c; Mca, metacaspase (yeast caspase); Bir, baculovirus IAP repeats; Ybh3p, yeast BH3-only protein; Nma111, nuclear mediator of apoptosis.

Release of cytochrome c and cleavage of caspases are two hallmarks of the apoptotic pathway in higher eukaryotes. Although there is no indication that the apoptosome protein complex does exist in yeast, release of cytochrome c upon induction of cell death has been demonstrated [131, 132]. Additionally, caspase activity has also been reported in yeast cells which

express a metacaspase, known as Yca1/Mca. Yca1 and the other caspases have slightly different cleavage capacities [133], but common substrates have been identified like GAPDH [134, 135].
As mentioned before, two protein families, BCL2 and IAPs, are known to regulate this apoptotic branch in metazoan. Although these families have a much more elaborated role in metazoan, two proteins have been identified in yeast to mediate apoptosis, Bir1p and Ybh3p (Fig. 4) [136, 137]. Bir1p, a homologue of the IAP family, is a cytosolic and nuclear protein mediating the sensitivity of yeast cells to apoptosis. Overexpression of Bir1p in yeast prolongs lifespan and reduces apoptotic-like cell death. Similar to metazoan, the IAP protein can be inhibited by Htra2/Nma111, which is located in the nucleus instead of the mitochondria [129, 136].
Ybh3p is a homologue of the BCL2 family and only recently identified. Ybh3p shows several resemblances with the human equivalents, such as BAX. Ybh3p can interact with BCL-xL and is translocated to the mitochondria upon lethal stimuli mediating apoptosis (Fig. 4). Deletion of *Ybh3p* prolongs lifespan and displays reduced sensitivity towards apoptosis [137].

Despite the much more sophisticated and large protein family regulating apoptosis in metazoan, the basic features are conserved between yeast and the higher eukaryotes. The absence of an extensive BCL2 family in yeast, does not necessarily implicate the absence of the whole pathway. Numerous examples exist which demonstrate that the human BCL2 family acts on conserved mitochondrial substrates in yeast corresponding directly to their homologues in human cell lines. Transformation of *BAX* for example, induced release of cytochrome c, ROS production and phosphatidylserine externalisation in yeast, suggesting the existence of similar pathways [125].

Whether a cell dies by apoptosis, necroptosis or autophagy depends on a variety of factors, such as cell type or the duration of stimuli. Another decisive factor is the level of cellular ATP [138, 139]. High levels of ATP will enable an apoptotic form of cell death, whereas low levels will stimulate cells to undergo necroptosis. Changes in the cellular ATP level will switch the cell death mode. Because the mitochondria are the main site of ATP

production, they will play a crucial role in the decision of the type of cell death. Besides the level of ATP, a variety of other factors will determine the cell death mode and a complex relationship exists between necroptosis, apoptosis and autophagy. Crosstalk between either of those different forms has been described and they can cooperate, antagonise or assist each other [70, 140, 141].
In yeast, the different modes of cell death are also highly interconnected and a regulated switch in cell death mode from apoptosis to necrosis has been described mediated by autophagy. This switch depends on various factors, and one determining factor is a concentration dependent shift, as described for acetic acid, H_2O_2 and pheromones. Where low doses trigger apoptosis, higher concentrations rather induce necrosis [50, 79, 125, 142]. The nature of nutrient deficiency can also determine the type of cell death which is additionally associated to the autophagic processes. During nutrient starvation, deletion of specific autophagic genes, such as *ATG8* or *ATG11* determines whether yeast is more susceptible to respectively necrosis or apoptosis [143].

Due to the complex crosstalk between the different cell death modes both in yeast and metazoan, it is very difficult to assign a single type of cell death to a certain cell death stimulus, such as *DFNA5*. Because mitochondria are central organelles important during different cell death processes, several aspects of the mitochondria will be highlighted with the emphasis on their role in cell death. Mitochondrial proteins which are released during cell death, their dynamics and morphology, and the mitochondrial permeabilisation complex essential for the execution of apoptosis, will be described in this chapter. As yeast was used in this thesis to study the DFNA5-related apoptotic pathway, a comparison is made between metazoan and yeast.

1.3 The central role of mitochondria in cell death

Mitochondria are the main producers of cellular ATP and this is mediated by oxidative phosphorylation. They serve as docking sites for different proteins thereby integrating several cellular responses [91, 131, 144-146]. Due to this

cooperation, mitochondria are central players in the decision of cell's fate for either survival or cell death. It is therefore essential to maintain intact mitochondria as disintegration would have a detrimental effect on the cellular energy supply.

Due to their role as energy power houses in the cell, mitochondria have long been considered to be closely related to cell survival. The determination of the mitochondrial localisation of many apoptosis-related proteins led to the discovery that the mitochondria are not only involved in cell survival but also associated with cell death [145-147]. Since then, a lot of research has been performed on different mitochondrial aspects related to cell death. Especially during apoptosis, the internal apoptotic pathway in particular, mitochondria are essential modulators for the proper execution of cell death. Several features of the mitochondria, such as the mitochondrial dynamics and the mitochondrial permeabilisation process, play a key role in programmed cell death [132, 148-151].

This part of the introduction will first focus on the mitochondrial morphology, determined by mitochondrial fusion and fission, and the mitochondrial permeabilisation process. Mitochondrial dynamics mediate adaptation under various conditions regulating the energy production, which is essential for proper mitochondrial functionality. The permeabilisation of the mitochondria will release several apoptotic factors essential during cell death. It is crucial to keep both processes in balance as deregulation of either of them will have detrimental effects on cellular health.

1.3.1 Mitochondrial dynamics: Fission and Fusion

Mitochondria are very dynamic organelles continually fusing, dividing and moving within cells using a microtubule and/or microfilament platform. They can either form a long, dynamic interconnected network or distinct spherical organelles [152, 153]. Balancing this dynamic behaviour is crucial to maintain functional mitochondria and is closely related to cell death [154, 155]. The heterogeneous morphology of mitochondria depends on various conditions, such as their metabolic conditions, the energy status of the cell or the cell cycle stage. The major components contributing to mitochondrial fusion and fission have been evolutionary conserved between yeast and

higher eukaryotes. Although the core machinery of this mechanism might be conserved, their regulation is far more sophisticated and different as novel regulators have been identified in mammals [156, 157]. Due to this conservation, yeast was one of the most important models where the key players of this dynamic process were characterised.
The biological objective behind this dynamics is the same for yeast as for any other organism. The main purpose is to maintain mitochondrial quality by distributing and removing damaged mitochondrial components providing a defence mechanism [158]. Mitochondrial dynamics and cell death are strongly connected and reveal the state of health of the population [159]. Enhanced fusion for example, is related to metabolic active cells mediating efficient energy transfer in the cell [160, 161]. Fission on the contrary, contributes to mitophagy and plays a role during cytokinesis and apoptosis [10, 162-165].

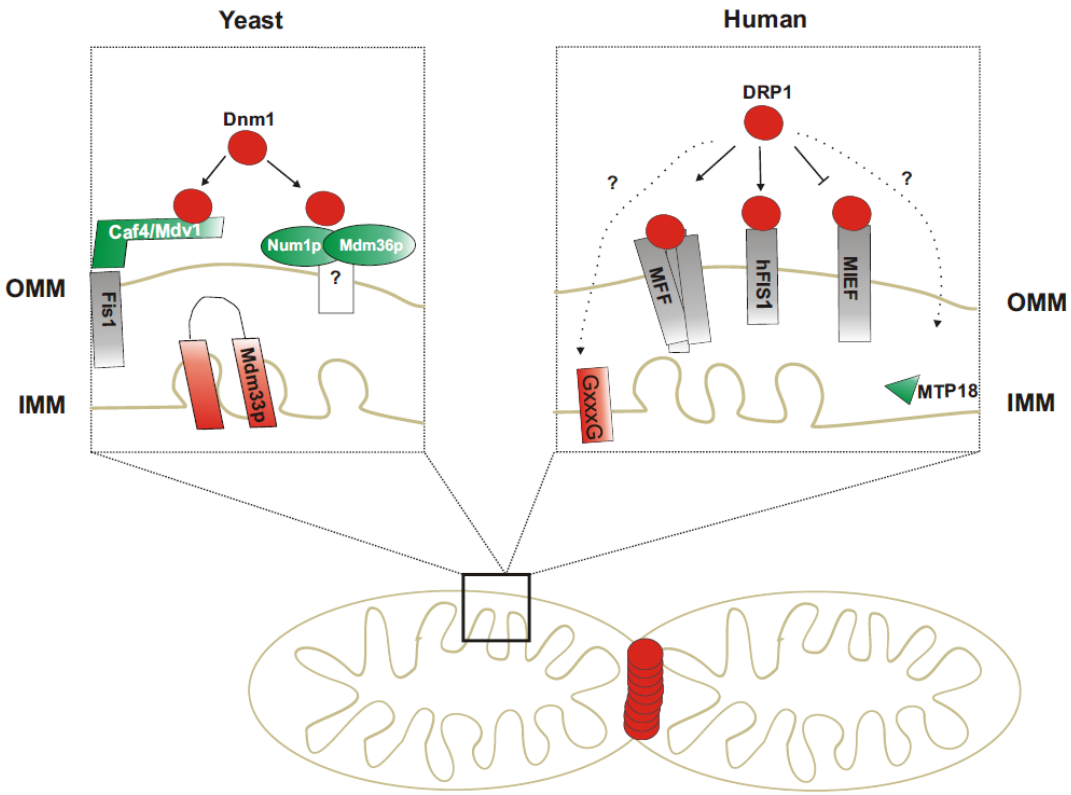
1.3.1.1 Fission machinery

The core machinery of fission contains two proteins: a dynamin-related protein (DRP1) and the (human) fission protein (hFIS1). Upon induction of apoptosis, DRP1 is recruited to the outer mitochondrial membrane (OMM) by hFIS1 which functions as a membrane receptor for DRP1 (Fig. 5) [166-170]. It has been shown that DRP1 can self-assemble into large multimeric ring-like structures in vitro, probably reflecting the constriction and fission of the mitochondrial membrane in vivo (shown at the bottom of Fig. 5) [167, 169-171].

In yeast, fission of the mitochondrial outer membrane is mediated by two additional proteins with no functional or structural homologues in multicellular eukaryotes, namely Mdv1 (Mitochondrial division) and Caf4 (CCR4-associated factor). They function as adaptor proteins mediating the recruitment of Dnm1 (the yeast homologue of DRP1) by Fis1 (the yeast homologue of hFIS1) (Fig. 5) [172-175]. Dnm1 and Fis1 share high functional similarity with their human equivalents as they both depend on GTP for energy supply during fission. Dnm1 is also able to form spirals around the mitochondria mediating their constriction and eventually scission upon GTP hydrolysis (shown at the bottom of Fig. 5) [171, 176]. Mdv1 and Caf4 are

cytosolic proteins associated with the OMM sharing structural similarity. In the presence of both Fis1 and Dnm1, they form a punctuate structure at the membrane mediating fission. Cooperation between Fis1, Dnm1 and Mdv1 or Caf4 is essential in order to drive correct mitochondrial fission, although Caf4 is dispensable for this process [172, 177].

Fig. 5: Comparison of the fission core machinery in yeast and human. '?' indicates currently unknown regulators or processes related to fission. Once DRP1/Dnm1 is recruited



to the mitochondrial outer membrane, DRP1/Dnm1 (red dots) will self-assemble into large multimeric ring-like structures, as shown below, mediating fission. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; Mdm30/36, mitochondrial distribution and morphology; Mdv1, mitochondrial division; MFF, mitochondrial fission factor; MIEF, mitochondrial elongation factor 1; Caf4, CCR4-associated factor; hFIS/Fis1, human fission protein; Num1p, nuclear migration protein; DRP1/Dnm1, dynamin-related protein; MTP18, mitochondrial protein.

Many studies have shown a correlation between apoptosis and mitochondrial dynamics. Mitochondrial fragmentation is a common hallmark of apoptosis, although it is not always present and does not necessary lead to apoptosis. Down-regulation of DRP1 by either siRNA or a selective inhibitor mdivi-1, inhibits mitochondrial fragmentation and inhibits cytochrome c release [150, 178]. Down-regulation of hFIS1 induces mitochondrial fusion and produces increased resistance to apoptosis. Furthermore, hFIS1-related apoptosis was characterised by release of cytochrome c and BAX recruitment [150, 179]. Also in yeast, a mutual relation exists between lifespan and the dynamics of the mitochondria. Dysfunctional mitochondria can lead to decreased lifespan, whereas elongated mitochondria enhance longevity [180, 181]. The same outcome as in mammalian cell lines was seen upon deletion of several yeast fusion and fission genes. Deletion of either *Dnm1* or *Mdv1*, two fission genes, increased resistance to apoptotic stimuli, such as acetic acid. Deletion of Fis1 however, reduced viability in yeast cells and it was suggested that during cell death Fis1 inhibits the Dnm1-mediated fragmentation [149]. Furthermore, down-regulation of several fission proteins, such as Mdv1, Fis1 and Caf4, was seen upon calorie restriction, a mechanism known to prolong lifespan in yeast [182].

1.3.1.2 Fusion machinery

In mammalian cell lines, mitochondrial fusion is mediated by three large GTPases, Mitofusin 1 and 2 (MFN1/2), and Optic Atrophy 1 (OPA1) (Fig. 6) [183, 184]. Two outer and two inner membranes of adjacent mitochondria need to be tethered to achieve mitochondrial fusion. Alternative splicing of OPA1 generates transcripts of different length necessary to mediate efficient mitochondrial fusion of the inner membrane [185-187]. MFN1 and MFN2 mediate mitochondrial outer membrane fusion, although they have a slightly different function. Only MFN1 is essential for the initiation of membrane tethering [183, 188, 189], while MFN2 acts more downstream of the initial process [190]. MFN2 has a broader spectrum of functions, one of them is related to the tethering of the ER to the mitochondria [190]. Recently a novel mitofusin-binding protein (MIB) was identified, which interacts with MFN1, thereby negatively regulating its function (Fig. 6) [191].

The core machinery for fusion in yeast exists of three proteins: Mgm1 (the yeast homologue of OPA1), Fzo1 (the yeast homologue of MFN1/2) and Ugo1 (Fig. 6) [192-194]. The function and localisation of these proteins are similar in yeast and human. Mgm1 plays a key role in the inner mitochondrial membrane (IMM) fusion. In contrast to OPA1, Mgm1 has only two splice isoforms, a short isoform present in the intermembrane space and a longer form located at the IMM. Fzo1, located in the OMM, is responsible for the formation of transcomplexes enabling tethering of adjacent mitochondria [193, 195]. Ugo1 is the third component of the yeast fission machinery and does not have a homologue in higher organisms. Ugo1 binds directly to Fzo1, by its cytosolic part, and to Mgm1, via its intermembrane domain, creating a bridge coordinating efficient fusion (Fig. 6) [192, 196].

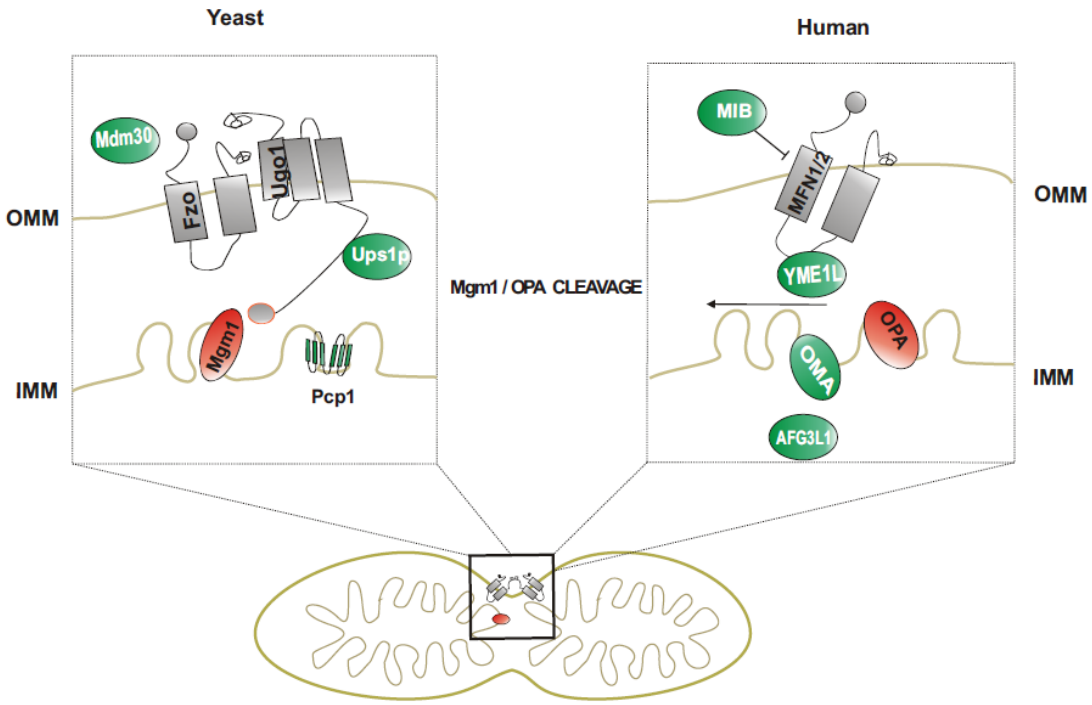


Fig. 6: Comparison of the fusion core machinery in yeast and human. Interaction of Fzo1/MFN1 of adjacent mitochondria leads to fusion of the OMM of opposing mitochondria. Fusion of the IMM is mediated by Mgm1/OPA. Ups1p, Pcp1p, AFG3L1, OMA and YME1L are regulators of Mgm1/OPA cleavage. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; Fzo1, fuzzy onion 1; OPA1, optic atrophy 1; Pcp1p, processing of cytochrome c peroxidase; MFN1/2, mitofusin 1 and 2; Mgm1, mitochondrial genome maintenance; MIB, mitofusin-binding protein; Mdm30, mitochondrial distribution and morphology; Ugo1, means fusion in Japanese; Ups1, unprocessed; OMA1/MPRP-1, metalloprotease-related protein-1; YME1L, yeast mitochondrial escape; AFG3L1, ATPase family gene-3 yeast-like-1.

The association between mitochondrial fusion and cell apoptosis has also been extensively described. Overexpression of one of the fusion proteins, MFN1 or MFN2, reduced the sensitivity to apoptosis by inhibiting cytochrome c release, whereas silencing enhanced the sensitivity to apoptotic triggers [197, 198]. A direct link between fusion and apoptosis was also shown for OPA1. Loss of OPA1 or mutations in functional domains of OPA1 both induced apoptotic cell death, whereas OPA1 expression protected cells from apoptosis [199, 200]. Overall, the balance between

fusion and fission is shifted during apoptosis, resulting in either excessive fission or insufficient fusion.
Besides modulating mitochondrial fragmentation, OPA1 also mediates cristae remodelling in the IMM enabling cytochrome c release [201, 202]. Under normal circumstances, OPA1 stabilises the cristae junctions by wrapping OPA1 oligomers around the junctions. Disruption of OPA1, by tBID or PARL (Presenilin-associated rhomboid-like) cleavage, mediates fusion of the cristae and opens these junctions, releasing cytochrome c [200, 201, 203].

1.3.1.3 Regulation of mitochondrial dynamics: yeast versus human

Fission

Although the two central components of fission are evolutionary conserved between yeast and human, the interaction partners assisting the core elements are functionally very different. Besides co-factors regulating the dynamics in vertebrates, post-translation modifications, including phosphorylation ubiquitination, phosphorylation and sumoylation, regulating either DRP1 or hFIS1, have been described. They can inhibit, activate or give regulatory feedback controlling mitochondrial fission [166, 204-206].

Additionally, multiple receptors have been identified regulating the recruitment of DRP1 to the mitochondria. Besides hFIS1, a mitochondrial elongation factor 1 (MIEF1) [207, 208] and a fission factor 1 (MFF) [209], two proteins with no yeast homologue, regulate fission (Fig. 5). MIEF1 prevents fission by sequestering DRP1 inhibiting their binding to GTP. In contrast to MIEF1, the interaction of MFF1 with DRP1 stimulates mitochondrial fission. MFF1 mediates DRP1 recruitment to the mitochondria through interaction by its N-terminus. Nine isoforms of MFF1 have been identified, supporting the more complex regulation of fission in human compared to yeast [210-213]. Besides these two main regulators, a variety of other smaller players could influence mammalian fission, including endophilin B1 [214], GDAP1 (ganglioside-induced differentiation-associated protein 1) [215] and

DDP/TIMM8a [216]. In order to achieve correct mitochondrial fission, coordination between the IMM and the OMM is needed. Two co-factors mediating this interplay have been proposed, namely MTP18 (mitochondrial protein) [217, 218] and MTGM (mitochondrial targeting GxxxG motif protein) [219] located in respectively the intermitochondrial space and at the IMM (Fig. 5). Reduction of either MTP18 or *MTGM* expression resulted in a highly interconnected mitochondrial network. Although a yeast homologue of MTGM has been identified, namely Mgr2 (mitochondrial genome required 2), its contribution to the mitochondrial dynamics in yeast is unknown [220].

For yeast, no post-translational modifications have been identified to influence fission, but a genome-wide screen on yeast deletion mutants did reveal three proteins to be involved in the regulation of mitochondrial fission. Although not essential for fission in yeast, Num1p interacts with Dnm1 stabilizing or recruiting Dnm1 to the mitochondria (Fig. 5) [221]. Mdm36p (mitochondrial distribution and morphology) is an extra factor contributing to this complex as this interaction is abolished in yeast cells lacking Mdm36p (Fig. 5) [222]. Inner membrane fission is regulated by Mdm33p, an integral mitochondrial inner membrane protein (Fig. 5). Deletion of Mdm33p led to the formation of interconnected mitochondria, whereas overexpression led to the aggregation of mitochondria [223].

Fusion

Similar to fission, differences regarding the regulation of the fusion process do exist. Regulators of the human mitochondrial dynamics can either mediate the processing of OPA1 or MFN1/2 degradation. Various proteases have been characterised to enable OPA1 cleavage, either residing in the inner membrane (OMA1/MPRP1) [224, 225], the matrix (AFG3L1 and AFG3L2) or the intermitochondrial space (YME1L) (Fig. 6) [186, 187, 226]. Although some of them do have yeast homologues, such as Oma1p and Yme1p, their contribution to the processing of yeast Mgm1p is largely unknown [227, 228] (Fig. 6). The activity of MFN1/2 is regulated by a variety of different proteins that mediate their degradation. Some proteins know to be involved are stomatin like protein-2 (Stoml2), mitochondria-associated

phospholipase D (mitoPLD), Mfn-binding protein (MIB) (Fig. 6) and many others, although their exact role in mediating fusion remains obscure [191, 229, 230].

In yeast, three major regulators of fusion have been identified mediating either Fzo1 or Mgm1 similar to the mammalian regulation. Mdm30p, of which no homologue has been identified in mammals, helps to maintain fusion competent yeast cells by regulating the Fzo1 level (Fig. 6) [231]. It is part of an ubiquitination and degradation complex which controls the turnover of Fzo1 after GTP hydrolysis. Two others mediate the processing of Mgm1. Pcp1p, located at the IMM, generates the short isoform of Mgm1 and Ups1p mediates this Pcp1p-dependent cleavage of Mgm1 (Fig. 6) [232, 233]. Both proteins are essential for maintenance of the mitochondrial morphology and have functional equivalents in mammals being respectively PARL and PREL1, although PARL seems to have only a minor role in the human fusion process in contrast to Pcp1p [203] (Fig. 6).

Conclusion

This pool of regulators reflects the complexity of mitochondrial dynamics. Although comparison of the core machinery of mitochondrial dynamics points to the conservation of this fundamental process, it is not unexpected that the fine-tuning has diverged over time. As mitochondrial dynamics have a major impact on numerous cellular processes, more sophisticated machinery is required in certain specialised cell types and this needed some adaptation in higher eukaryotes. Although most of the players have a homologue (Dnm1/DRP1, Fis1/hFIS1, Fzo1p/MFNs and Mgm1p/OPA1), the amino acid sequence revealed some major differences implying functional diversity. Not all homologues are functionally interchangeable; some require certain co-factors which are not conserved, such as Caf4, Mdv1 or MFF1, which further increases the complexity of the mitochondrial dynamics. The preservation of the fundamental elements however, supports the value of yeast to reveal the essential components of cellular processes.

1.3.2 Mitochondrial permeability transition

One of the hallmarks of apoptotic cell death is the permeabilisation of the mitochondria, a highly complex and tightly regulated mechanism. Several models have been proposed but there is no consensus and the exact mechanism still needs to be clarified. Permeabilisation is a critical event during apoptosis, mediating the release of several mitochondrial apoptotic proteins crucial for the execution of apoptosis [234, 235].

Mitochondria consist of two compartments: the matrix, enclosed by the IMM, and the intermitochondrial space enclosed by the OMM. Both the IMM and OMM show signs of permeabilisation during apoptosis due to the formation of a scaffold structure at the contact sides of both membranes, called the permeability transition pore complex (PTPC) [236]. The PTPC is a large, high conductance protein complex comprising two main components, the voltage-dependent anion channel (VDAC) located at the OMM, and the adenine nucleotide translocase (ANT) present at the IMM, with the addition of smaller modulators, such as cyclophilin-D (CypD) and hexokinase (HK) (Fig. 7) [235, 237-239].

Several models have been proposed to explain the mitochondrial permeabilisation. Some concern the PTPC, others consider the rupture of OMM due to the increase of the IMM permeability and yet some even declare the existence of an unknown channel formed by aggregation of membrane proteins. Until now, there is no consensus and recently the F_1-F_0 ATP synthase is even suggested to be a main player [240, 241]. A key event, where most of the investigators seem to agree on, is that mitochondrial permeabilisation is stimulated by an elevated Ca^{2+} concentration leading to several physiological cellular modifications [242-244]. The mitochondrial permeabilisation is characterised by mitochondrial swelling, loss of the mitochondrial membrane potential ($\Delta\Psi$), increased permeability of the IMM with a cut-off of 1500 kDa and the release of several apoptotic factors. The following part will describe the components known to be related to mitochondrial permeabilisation, their regulators, recent advances and the conflicting results.

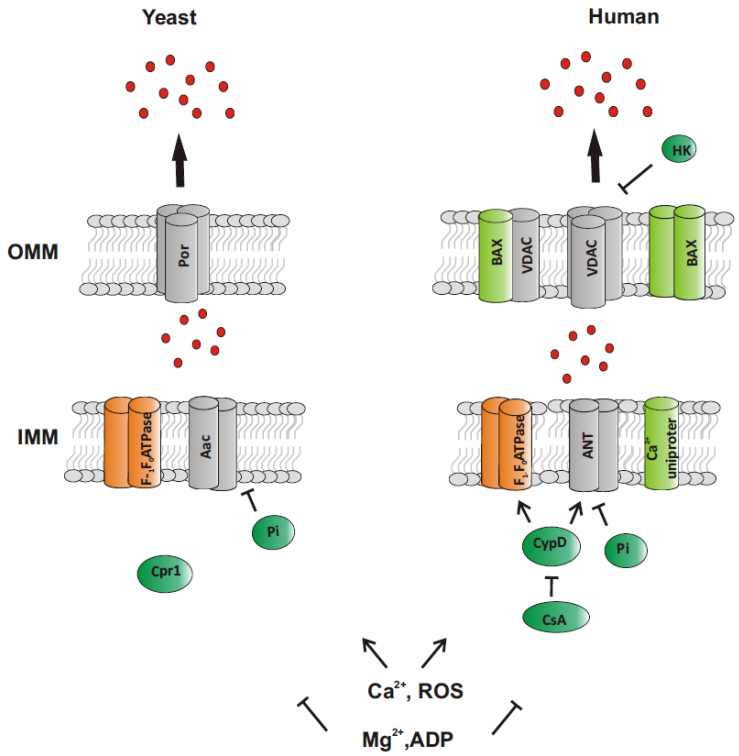


Fig. 7: Comparison of the mitochondrial membrane permeabilisation process in yeast and human. Several protein complexes, present at both the OMM and the IMM, are responsible for the permeability process. This can be mediated by several components, such as ROS, ADP, Mg^{2+} and Ca^{2+}. VDAC/Por, voltage-dependent anion channel; ANT/Aac, adenine-nucleotide translocase; CypD/Cpr, cyclophilin-D; Pi, phosphate; CsA, cyclosporine A; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; ROS, reactive oxygen species; HK, hexokinase. Apoptotic factors are indicated by red circles.

1.3.2.1 Metazoan

Mitochondrial outer membrane permeabilisation (MOMP) leads to the release of several apoptotic factors stimulating cell death. The VDAC is a mitochondrial membrane protein, present at the OMM, responsible for an efficient exchange of metabolites between the mitochondria and the cytosol (Fig. 7) [245-247]. The role of VDAC in the permeabilisation of the mitochondria, has been established by several studies. Whereas silencing of the *VDAC* channel resulted in reduced cytochrome c release from the mitochondria and inhibition of caspase-3 activation [248], overexpression of VDAC was associated with Δψ collapse [249].

The opening of the VDAC channel, formed by oligomerisation of the transmembrane protein [250], is regulated either directly or indirectly by various components such as HK [251], Ca^{2+} [252] and BCL2 family proteins (Fig. 7) [147, 253]. Binding of HK-I and HK-II to VDAC, both catalysts of the glycolysis, results in closure of the VDAC channel. HK interaction would prevent the formation of the VDAC-BAX complex and hence protects the cell from mitochondrial apoptotic cell death [251, 254].

Permeabilisation of the mitochondria during cell death is characterised by the collapse of the ΔΨ, which is maintained over the IMM. The electron transport chain complex in the IMM transfers protons from the IMM to the intermitochondrial space, resulting in the proton gradient used for oxidative phosphorylation. Dissipation of this potential is a result of the sudden increase in the permeability of the IMM to small ions and water, known as mitochondrial permeability transition (MPT) [255, 256]. ANTs are considered to be important for this permeabilisation over the IMM as they transport metabolites across the IMM and contribute to the exchange of ATP for ADP (Fig. 7). ANT proteins are transmembrane proteins capable of forming channels [236, 257, 258] (Fig. 7). The role of ANT in apoptosis was supported by the finding that binding of ANT inhibitors, such as bongkrekic acid, mediated the induction of apoptosis [259].

Despite the evidence for both protein complexes in mitochondrial permeabilisation [236, 239, 260, 261], the necessity of these complexes has been controversial as they seem to be dispensable for mitochondrial

transition. Deletion of two ANT isoforms in mice for example, still resulted in mitochondrial permeabilisation, despite an increased resistance to cell death [262]. Similar to ANT, VDAC null mice displayed no differences in cytochrome c release and caspase cleavage compared to wild-type mice [263, 264]. It is clear that both complexes do play a role in the mitochondrial permeabilisation. Their necessity for efficient transition however, is still a matter of debate and is uncertain at this moment. In addition to VDAC and ANT, another player of the mitochondrial permeabilisation complex, CypD, has been accepted as an important regulator in pore opening.

Role of Cyclophilin-D

CypD belongs to the cyclophilin family and is, besides its role in cell death, crucial for protein folding [265]. The recognition of CypD as a key regulator of mitochondrial transition was determined by binding of cyclosporine A (CsA), a specific inhibitor of the cyclophilin family, which inhibited pore opening (Fig. 7) [266]. CypD resides in the matrix and binds to the IMM during mitochondrial transition. It has been shown that CypD can interact with either ANT or VDAC [267]. Similar to VDAC and ANT, there is some controversy about the role of CypD in MPT. Although no MPT was noted in a CypD knock-out mouse model stimulated by Ca^{2+}, it has been reported that even without CypD there is still some level of mitochondrial transition provided that a higher Ca^{2+} concentration is used to stimulate such as permeabilisation [268-270]. Although the exact role remains to be clarified, it is certain that CypD at least sensitises pore permeabilisation for Ca^{2+}-induced MPT.

1.3.2.2 Yeast

Besides the mammalian mitochondrial transition pore, several mitochondrial pores have been identified in other eukaryotes including yeast. The physiological role and the regulation of these channels are still a matter of debate and conflicting results were reported [271, 272]. Despite the discrepancies, several studies have shown that the yeast mitochondrial transition pore (yPTP) is indeed related to the mammalian permeability transition pore (mPTP). Yeast displays several characteristics that suggest the existence of an yPTP. Isolated mitochondria show signs of mitochondrial

swelling, similar to mammalian mitochondria, and the release of solutes was demonstrated [272, 273]. Additionally, the release of cytochrome c was observed in response to a variety of cell death triggers, such as H_2O_2, acetic acid and BAX [131, 132, 271].

Analogous to human mitochondrial permeabilisation, similar protein complexes have been identified in yeast. Shimizu *et al.* demonstrated the role of the yeast homologue of VDAC, Porin (Por), in MPT as VDAC deficient yeast strains were resistant to cytochrome c release stimulated by BAX (Fig. 7) [253]. Since the unravelling of the Por sequence in yeast [274], several researchers already investigated the structural and functional homology between Por and VDAC. Despite the low degree of conservation of the primary sequence, the yeast and human VDAC show clear structural and functional similarities illustrated by comparable gating properties such as, the conductance of the pore and the pore size [273, 275, 276]. Additionally, the Aac proteins, the yeast homologues of the ANT genes, appear to be essential for the release cytochrome c, in yeast (Fig. 7) [128, 132].

Despite the conservation of the main players, some differences between the yPTP and mPTP were also observed. The main difference is the sensitivity of the pore to CsA. As mentioned before, CsA is an inhibitor of the mPTP by interaction with CypD [266, 277]. Although a yeast homologue of CypD does exist, Crp1, there is no apparent relationship to the yPTP [267, 272]. Recently however, it was discovered that not CsA, but inorganic phosphate (P_i) is the actual pore inhibitor and that binding of CsA to CypD unmasks an inhibitory site to Pi. CsA has been shown to indeed inhibit PT but only in the presence of Pi, suggesting that Pi and not CsA is the actual inhibitor [278, 279]. It should be noted that even in the presence of CsA, pore opening is still possible although higher concentrations of Ca^{2+} are needed. As such, CsA is not a true blocker but rather desensitise pore opening. As yPTP is indeed sensitive for Pi, this could explain the interspecies discrepancy seen between mPTP and yPTP [272, 280]. Based on these observations, Pi could be the common feature of PT in all organisms (Fig. 7). The role of Pi in PT regulation is however not certain as independence of CsA on Pi has been described recently [281, 282].

As already mentioned above, a feature of pore opening where most of the researchers seem to agree on is the susceptibility to Ca^{2+} concentration [242-244]. Elevated Ca^{2+} concentration stimulates pore opening, which is the second unifying feature between yPTP and mPTP. Although contrasting results in yeast do exist and yeast cells lack a mitochondrial Ca^{2+} uniporter necessary for rapid Ca^{2+} uptake, yPTP can be induced by Ca^{2+} on condition of low Pi concentration [242, 272]. A last important common feature is the recent elucidated role of the ATP synthase in PTP. Recently, it was shown that the F_0-F_1 ATP synthase is able to form channel-like dimers in lipid bilayers (Fig. 7) [240]. This ATP synthase dimer has properties matching those of PTP, being Ca^{2+}-sensitive, and has similar channel conductances. Additionally, it was found that CypD binds to the F_0-F_1 ATP synthase hereby stimulating pore opening. That F_0-F_1 ATP synthase dimer is the actual pore, is further supported by a specific inducer of dimerisation, Bz-423. Bz-423 shares the same binding site as CypD and can trigger the channel activity of this ATP synthase [240]. Additionally, the channel forming capacity of the ATP synthase has also been demonstrated in yeast, resembling features of the mammalian ATP synthase dimers being activated by Ca^{2+} and inhibited by Mg^{2+} and ADP (Fig.6) [241].

1.3.2.3 Regulation of mitochondrial permeabilisation

Despite the conservation of different components involved in mitochondrial permeabilisation, some discrepancies regarding the regulation do exist. One of the main differences between yeast and higher eukaryotes is the presence of the BCL2 family. These are one of the most important regulators regarding mitochondrial cell death, but seem to be completely absent in yeast. Only recently one yeast homologue of this family, namely the yeast BH3-only protein, Ybh3p, has been identified [137].

In higher eukaryotes, the BCL2 family protein, is far more sophisticated and can be divided in three main groups as mentioned previously. Although the main function of the BH3-only proteins is to modulate both the pro- and the anti-BCL2 proteins, they can also interact with either VDAC or ANT mitochondrial proteins thereby mediating the release of several mitochondrial components (Fig.6) [123, 283].

Evidence exists that either anti- and pro-apoptotic BCL2 proteins can regulate the VDAC and the ANT channel conformation [147, 284, 285]. The pro-apoptotic BAX protein for example translocates from the cytosol to the mitochondria upon induction of apoptosis and binds to VDAC leading to the release of cytochrome c [147]. The anti-apoptotic BCL-xL however, reduces channel conductivity by binding to VDAC [286-288]. Similarly to the VDAC, ANT can be regulated by either pro- and anti-apoptotic BCL2 proteins acting respectively as facilitators and inhibitors of channel formation [257, 284, 285]. Although only one BCL2 member has been identified in yeast, Ybh3p shows several resemblances with the human equivalent BAX. Similar to BAX, Ybh3p translocates to the mitochondria upon induction of apoptosis [137]. Furthermore, Uth1p, a mitochondrial outer membrane protein, was needed for Ybh3p-mediated cell death, analogous to BAX-mediated cell death in yeast [289]. Additionally, it has been shown that Ybh3p can interact with BCL-xL thereby inhibiting its pro-apoptotic role and suppressing its lethal effects (Fig. 7) [137, 289, 290].

The BCL2 proteins can also form channels independent of VDAC and ANT. Once present in the OMM, large homo- or hetero-oligomer complexes of BCL2 proteins will lead to channel formation and cytochrome c release [291, 292]. This conformational change will generate different channel complexes, such as dimers, trimers or tetramers, all contributing to the mitochondrial permeabilisation (Fig. 7). Although their exact contribution still remains controversial, it was demonstrated that pro-apoptotic BCL2 proteins can form higher order oligomer membrane formations in vitro [293]. This indicates their capacity to form channels and hence contributes to the mitochondrial permeabilisation process and the release of apoptotic factors [294-297].

Despite the absence of an extensive BCL2 family in yeast, the pathways by which they exert their function seem to be conserved in yeast. This implicates that the human BCL2 family acts on conserved, mitochondrial substrates present in yeast, which will be shown by a few examples described hereafter. Transformation of *BAX* in yeast induced release of

cytochrome c, ROS production, phosphatidylserine externalisation, DNA fragmentation and chromatin condensation, suggesting the existence of conserved pathways [125, 298-301]. Similar to human cell lines, BAX was closely connected to the mitochondria, which was indeed essential for the BAX-induced toxicity in yeast. It was shown that in yeast, BAX has the capacity to form channels in the OMM and that either VDAC or Aac proteins are essential for the BAX-induced cell toxicity, although conflicting results exist [253, 257, 302, 303]. Many of these events can be suppressed by co-expression of either BCL-xL or BCL2, confirming the conservation of its protective role upon cell death in yeast [301, 304]. Finally, it was demonstrated that, despite the lack of obvious homologues, some aspects of the BCL2 proteins seem to be present in other proteins. A good example hereof is Fis1, which was demonstrated to have BCL2-like anti-death activity [149]. During normal growth, Fis1 is required for mitochondrial fragmentation, but during cell death Fis1 protects cells from Dnm1-mediated fragmentation and loss of functional mitochondria. In this study, Fis1 formed pores in lipid vesicles, similar to BCL-xL, and BCL-xL or BCL2 could restore viability in Fis1-deficient yeast cells. Based on these data it is possible that Fis1 contains two functions, the protective role of BCL2 and a fission function of hFIS1 which are carried out by two different proteins in higher eukaryotes [149]. This further supports the existence of conserved pathways between yeast and higher eukaryotes.

Until today, there is no consensus about mitochondrial permeabilisation and alternative models are suggested. One model suggests that BAX would only regulate pre-existing mitochondrial membrane channels, such as the PTPC as describe above. Another model for OMM permeabilisation is based on osmotic swelling due to a sudden increase in the IMM permeability. This permeabilisation drives water and small ions into the matrix, leading to swelling of the matrix and eventually to rupture of the OMM [305].

Despite the incomplete understanding of either the yPTP or the mPTP, the importance of mitochondrial permeabilisation in cell death has been demonstrated. The ability to specifically regulate pore opening in yeast,

supports the existence of yPTP. Although discrepancies between yPTP and mPTP in pore regulation do exist, the main players and the global mechanisms are conserved. Once the mitochondrial permeabilisation process is initiated, a variety of mitochondrial intermembrane proteins are released into the cytosol all contributing to the cell death process.

1.3.2.4 Release of mitochondrial proteins upon permeabilisation

One of the first proteins which is released upon activation of the mitochondrial pathway is cytochrome c, initially identified as APAF-2. The release of cytochrome c is considered as a point of no return in the apoptotic cascade [306, 307]. Cytochrome c resides in the cristae of the IMM and was first identified to play a role in the electron transport chain complex. Cytochrome c is bound by cardiolipin at the cristae of the IMM. Conformational changes in the cardiolipin-cytochrome c interaction and remodelling of the cristae are needed prior to its translocation [308]. Increase in H_2O_2 levels, cytosolic Ca^{2+} and in ATP level can all modify the oxidation status of cardiolipin and will lead to the dissociation of cytochrome c from the IMM, a required step for the induction of apoptosis [309, 310]. Once mobilised, cytochrome c is translocated to the cytosol.

As the release of cytochrome c is irreversible, this process is controlled at several levels. First, the remodelling of the cristae is regulated by different proteins that are involved in the mitochondrial morphology. OPA1, as mentioned before, is one of the fusion proteins and is known to regulate the width of the cristae and to sequester cytochrome c. Under normal circumstances, OPA1 stabilises and narrows these junctions by forming oligomers around them [200, 201, 311]. In addition to OPA1, DRP1 and MFN1/2 have also been linked to cristae remodelling, by respectively promoting and blocking cytochrome c release [312-314].

Besides their role of the mitochondrial fusion and fission machinery, the BCL2 proteins, especially the pro-apoptotic and BH3-only proteins, also play a role in regulating cytochrome c release. As described above, certain BCL2 proteins have pore-forming capacities, disrupting the OMM. Activated Bid (tBid), a BH3-only protein, is known to interact with cardiolipin. Binding of

tBid to cardiolipin followed by oligomerisation of BAX/BAK both contribute to the release of cytochrome c [315, 316]. Despite the contribution of numerous mitochondrial proteins in the release of cytochrome c, recent results suggest that cytochrome c can contribute to its own escape by inducing discrete pores in the OMM in the absence of other apoptotic factors due to the cardiolipin-cytochrome c interaction [317] (Fig. 3).
In addition to cytochrome c, other mitochondrial proteins are released during mitochondrial permeabilisation, such as Smac/DIABLO, HtrA2/OMI, EndoG and AIF [92, 95, 96]. The two last are considered to induce apoptosis through a caspase-independent pathway. Once released from the mitochondria, they are translocated to the nucleus, which results in DNA fragmentation [110, 111, 146].

Until today there is no indication that a yeast apoptosome does exist. Yeast homologues of cytochrome c, such as Cyc1 and Cyc7, have been characterised but their contribution to the yeast metacaspase is uncertain at this moment. Nevertheless, release of cytochrome c upon induction of cell death has been demonstrated in yeast [132]. Additionally, as already mentioned, other apoptotic factors are also released in yeast from the mitochondria upon cell death induction. Translocation of Nuc1 and Aif from the mitochondria to the nucleus has been demonstrated, where it has a homologous function, namely fragmentation of DNA (Fig. 4) [127, 128]. As such, a caspase independent pathway seems to be present in yeast a well.

1.4 Objectives of this research

The general objective of this project was to further elucidate the molecular mechanisms induced by mutDFNA5. The function of DFNA5 remained unknown for a long time due to the fact that *DFNA5* has no clear structural or functional similarities with other genes. Gregan *et al.* (2003) demonstrated for the first time the toxic effect of mutDFNA5 in fission yeast [39]. Recently, functional studies on DFNA5 revealed a direct link to apoptosis in human cell lines [17]. This was the first indication that the DFNA5-related mechanisms have some conservation among different species. This observation led us to our first specific aim of this thesis.

1.4.1 Elucidation of the DFNA5-associated apoptotic mechanism in a yeast model

In order to unravel specific apoptotic mediators of the DFNA5 process in *Saccharomyces cerevisiae*, an apoptotic deletion strain collection was screened. This collection contained eighteen different deletion strains, strains in which one specific component, known to be related to apoptosis, is deleted. The first specific objective was to evaluate the growth defect of each of these strains upon mut*DFNA5* transformation, followed by the determination of the type of cell death by specific stainings. This growth defect was then compared to the growth defect of the wild-type yeast strain to determine the effect of the deleted gene. To further unravel pathways related to DFNA5-induced cell death, a transcriptomic analysis was performed to compare the expression profiles of wt*DFNA5* and mut*DFNA5* transformed yeast cells.

1.4.2 Investigation of the DFNA5-associated apoptotic mechanism in human HEK293T cells

As *DFNA5* is a human gene related to HL and cancer, the results obtained in the yeast model were investigated in human cell lines to evaluate whether the results obtained in yeast could be confirmed. We wondered whether yeast would be a good model to study DFNA-related mechanisms and therefore focused on several mitochondria-related aspects. In order to unravel specific DFNA5-related pathways, previously obtained

transcriptomic data of *DFNA5* in HEK293T cells were re-evaluated and additional functional studies were performed.

As mitochondria are central players in different types of cell death, several mitochondrial assays were performed to determine the role of this organelle in DFNA5-induced cell death. The mitochondrial permeabilisation, the mitochondrial structure and different mitochondrial proteins were investigated. A role for the mitochondria in DFNA5-associated cell death does not necessarily implicate apoptosis, therefore we wondered whether other types of cell death would be involved in the DFNA5 mechanism. Therefore, extra experiments were performed to study the role of DFNA5 in non-apoptotic forms of cell death.

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Part II

Results

Chapter II

The splicing mutant of the human tumour suppressor protein DFNA5 induces programmed cell death when expressed in the yeast *Saccharomyces cerevisiae*

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The yeast deletion strains used in this chapter were generated from the genome-wide yeast collection of the 'European Saccharomyces cerevisiae Archive for Functional analysis' (Euroscarf, Frankfurt, Germany). All the DFNA5 plasmid constructs used in this chapter and the experiments performed in HEK293T cells were made and performed by Dr. Ken Op de Beeck. Dr. Vanessa Franssens generated additional fluorescence data. The rest of the scientific experiments were performed by Sofie Van Rossom. All the co-authors contributed to the corrections and revision of the written report.

2 The splicing mutant of the human tumour suppressor protein DFNA5 induces programmed cell death when expressed in the yeast *Saccharomyces cerevisiae*

2.1 Abstract

DFNA5 was firstly identified as a gene responsible for autosomal dominant deafness. Later, it became clear that the protein also has a tumour suppression function and that it can induce apoptosis. Epigenetic silencing of the *DFNA5* gene is associated with different types of cancers, including gastric and colorectal cancers as well as breast tumours. We introduced the wild-type and mutant *DFNA5* allele in the yeast *Saccharomyces cerevisiae*. The expression of the wild-type protein was well tolerated by the yeast cells, although the protein was subject of degradation and often deposited in distinct foci when cells entered the diauxic shift. In contrast, cells had problems to cope with mutant DFNA5 and despite an apparent compensatory reduction in expression levels, the mutant protein still triggered a marked growth defect, which in part can be ascribed to its interaction with mitochondria. Consistently, cells with mutant DFNA5 displayed significantly increased levels of ROS and signs of programmed cell death. The latter occurred independently of the yeast caspase, Mca1, but involved the mitochondrial fission protein, Fis1, the voltage-dependent anion channel protein, Por1 and the mitochondrial adenine-nucleotide translocators, Aac1 and Aac3. Recent data proposed DFNA5 toxicity to be associated to a globular domain encoded by exon 2 to 6. We confirmed these data by showing that expression of solely this domain confers a strong growth phenotype. In addition, we identified a point mutant in this domain that completely abrogated its cytotoxicity in yeast as well as human HEK293T cells. Combined, our data underscore that the yeast system offers a valuable tool to further dissect the apoptotic properties of DFNA5.

Key words: DFNA5, *Saccharomyces cerevisiae*, yeast, cell death, apoptosis, hearing impairment, tumour suppressor

2.2 Introduction

Evasion of apoptosis is known to be an important factor in tumorigenesis, but the underlying mechanisms are often not well understood. Therefore, more research is required on the factors that govern cellular decisions between malignant outgrowth or programmed cell death, as this may eventually lead to the design of more efficient anti-cancer therapies [1-3]. Here, we describe our findings on the heterologous expression in yeast of a human protein that has an important role in controlling the switch between cell survival and cell death, *i.e.* DFNA5.

Supported by the observation that the exon 8 splicing mutant of DFNA5 (mutDFNA5), but not the wild-type allele (wtDFNA5), triggers cell cycle arrest when expressed in the fission yeast *Schizosaccharomyces pombe* [4], we decided to use yeast as a model to investigate the role of DFNA5 in more detail. We expressed wtDFNA5 and mutDFNA5 in the budding yeast *Saccharomyces cerevisiae* and analysed the repercussions on growth, oxidative stress and the induction of programmed cell death (PCD). We first evaluated the value of *Saccharomyces cerevisiae* as a model organism to unravel the mechanisms related to DFNA5.

Based on these results, we screened a collection of several apoptotic deletion strains (these are yeast strains deleted in one specific gene related to apoptosis) and compared the growth defect of *DFNA5*-transformed deletion strains to the background strain. Data obtained from the wild-type strain and a series of deletion mutants confirmed that mutDFNA5 strongly induces PCD, a phenomenon being dependent on mitochondrial integrity, but independent of the yeast caspase, Mca1. Several mitochondrial proteins were identified, namely the mitochondrial fission protein, Fis1, the voltage-dependent anion channel protein, Por1 and the mitochondrial adenine-nucleotide translocators, Aac1 and Aac3.

In addition, the region responsible for the induction of programmed cell death was assigned to the first globular domain of DFNA5, similar to which

was previously identified in HEK293T cells. Amino acid substitution of the second, highly conserved, hydrophobic amino acid reduced this cell death-inducing capacity, which proves that yeast is an ideal tool to identify point mutations. This underscores the feasibility of the yeast model for the unravelling of the cellular mechanisms of DFNA5.

2.3 Materials and methods

2.3.1 Strains, plasmids and growth analysis

In this study, we used the BY4741 wild-type strain [5] and isogenic deletion strains of the genome-wide collection (EUROSCARF, Frankfurt, Germany) lacking proteins involved in PCD as listed in Suppl. Table 2.1 The C-terminally HA-tagged full-length wt*DFNA5* and mut*DFNA5* cDNAs, the C-terminally EGFP fusion proteins and the wt*DFNA5* first and second globular fragments were isolated and amplified as previously described [4, 6] using the primers listed in Suppl. Table 2.2. All amplified products were ligated into the pYX212 plasmid using either *Eco*RI and *Bam*HI or *Eco*RI and *Sal*I restriction sites, thereby placing the inserts under expression control of the constitutive *TPI1* promoter. The mutants HCA-F2R and HCA-A3R at the N-terminal end of the first globular domain were generated by site-directed mutagenesis (Agilent, Santa Clara, USA) in combination with the custom designed primers listed in Suppl. Table 2.2. All constructs were verified by bidirectional sequencing on an ABI genetic analyser 3130xl (Applied Biosystems, Foster City, CA, USA). The construction of the plasmid for Mito-RFP was previously described [7]. Standard transformation techniques were applied [8] and all strains were grown at 30°C in a selective minimal medium containing 2% of glucose (SD-Ura). Growth profiles were determined in 96-well microtiter plates with continuous shaking at 30°C in a Multiskan GO spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Overnight cultures of at least three different transformants were diluted to start new cultures for growth analysis. Growth was monitored until the stationary phase was reached. Growth curves are depicted with scaled OD units and as the mean values of the transformants, with error bars representing standard deviations. The growth profiles of the strains expressing HA- or EGFP-tagged wtDNFNA5 or mutDFNA5 were compared to that of a control strain

transformed with either empty vector or a plasmid allowing for expression of EGFP. The differences in time required to reach half maximal optical densities (ΔT½) were calculated and used as standards for growth quantification. The difference in ΔT½ obtained for wtDFNA5 (ΔT½ wtDFNA5-Control) and mutDFNA5 (ΔT½ mutDFNA5-Control) in the BY4741 wild-type strain was used as reference and set as 100%.

Human Embryonic Kidney 293T cells (HEK293T) were subcultured in 60 mm dishes at a density of 2 x 10^6 cells in Dulbecco's modified Eagle's medium containing 4500 mg/l glucose supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all products from Invitrogen, San Diego, USA). The cells were incubated overnight at 37°C in a 5% CO_2 humidified environment. The plasmids used for wt*DFNA5* and mut*DFNA5* expression in mammalian HEK293T cells, after transfection with lipofectamine, were described before [6]. In addition, we used the pEGFP-N1 vector to construct plasmids for the expression of HCA-F2R and HCA-A3R mutants.

2.3.2 Western blot analysis

Yeast samples were grown in 3 ml cultures on selective medium and were harvested at an OD_{600nm} between 1.5 and 2.0. An equal amount of cells were taken and lysed by boiling for 15 minutes in SDS sample buffer [50 mM Tris (pH 8.0), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol]. Proteins were separated by standard SDS-PAGE and blotted onto PVDF membranes (Immobilon-P transfer membranes, Millipore, MA, USA). For immunodetection we used the primary rabbit anti-HA or anti-EGFP antibodies (Santa Cruz, CA, USA) and a secondary horse radish peroxidase (HRP) conjugated goat anti-rabbit antibody (Santa Cruz, CA, USA). The endogenous yeast alcohol dehydrogenase Adh2 served as internal standard. Membranes were developed using the ECL detection kit (Thermo Scientific, IL, USA).

2.3.3 Flow cytometric analysis of cell death, ROS accumulation and caspase activity

Tests for apoptotic and necrotic markers, using AnnexinV/Propidium Iodide (AV/PI) co-staining, as well as ROS-accumulation, using the superoxide-

driven conversion of non-fluorescent dihydroethidium (DHE) to fluorescent ethidium, were performed and quantified using BD Influx flow cytometry (BD Biosciences, New Jersey, USA) as described previously [9]. Yeast samples were harvested at different time points. Samples were collected at mid-exponential phase at an OD_{600nm} between 3.5 to 4.0, just after cells had traversed the diauxic shift (PD) at an OD_{600nm} between 6.0 to 7.0, and in stationary phase (ST) at an OD_{600nm} of 8.5 or higher. Analysis of the BD influx flow cytometry data was performed using the software program FlowJo (Tree Star Inc., Ashland, USA). Viability tests of the HCA-F2R and HCA-A3R mutants in HEK293T cells were performed on a FACScan flow cytometer (BD Biosciences, New Jersey, USA) after staining of the cells with PI. Cell viability was then determined as the ratio of cells showing no PI fluorescence to the total cell population.

A previously described protocol was used to measure the caspase activity [10]. Yeast cells grown on selective medium were harvested at an OD_{600nm} of approximately 4.5. A staining solution containing 10 µM FITC-VAD-FMK in PBS (CaspACE, Promega, WI, USA) was added to an amount of cells corresponding to an OD_{600nm} of 0.5 and incubated for 20 minutes at room temperature. After washing and suspension in 200 µl PBS flow cytometric analysis was performed using a 530/40 bandpass filter.

2.3.4 Fluorescence microscopy

Cells transformed with wt*DFNA5* or mut*DFNA5* fused to EGFP were grown until mid-exponential or post-diauxic phase as indicated and visualised using a Leica DM4000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were taken with a Leica DFC420C camera using the Leica Application Suite software. The percentages of post-diauxic cells with or without inclusions were determined by manual counting of at least 300 cells per sample.

Mitochondria were visualised by the expression of a mitochondria-targeted red fluorescent protein, Mito-RFP [7]. To stain the vacuolar membrane, cells were in the post-diauxic phase and incubated with FM4-64 ([N-(3-triethylammoniumpropyl)-4-(p-diethylamino phenylhexa-trienyl) pyridinium dibromide]; Molecular Probes, Eugene, USA) at room temperature for at least 30 minutes in a HEPES buffer containing 1% of glucose to facilitate the

uptake of FM4-64. To visualise the nucleus, we performed a 4',6-diamidino-2-phenylindole (DAPI) staining. The cells were harvested in the post-diauxic phase and incubated for 20 minutes in a phosphate buffer (0.04 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, 0.1 g/100 ml NaN_3) containing 50% ethanol. After washing with PBS, DAPI was added (1 µg/µl) and samples were incubated at room temperature for 15 minutes.

2.3.5 Statistical analysis

All experiments included biological replicates and the use of independent transformants. Statistical analysis was performed using unpaired *t*-tests or one-way ANOVA.

2.4 Results

2.4.1 Mutant DFNA5 induces apoptotic and necrotic cell death in yeast

To study the properties of human DFNA5 in a well-defined model, we expressed the cDNAs of wt*DFNA5* and mut*DFNA5* in the BY4741 wild-type strain. We used high-copy-number plasmids allowing expression of wtDFNA5 and mutDFNA5 as C-terminally HA-tagged proteins under the control of the strong constitutive *TPI1* promoter. For wtDFNA5, this resulted in good expression levels of the full-length protein, though we noticed that the protein was subject of proteolytic degradation as evidenced by the presence of discrete breakdown products upon western blot analysis (Fig. 1a). A lower expression level was obtained for mutDFNA5 and, interestingly, no major proteolytic fragments were observed in this case, even not when higher concentrations of protein extracts were loaded or when the exposure time of the immunoblots was increased. Growth analysis of the transformants revealed that the expression of wtDFNA5 had only a moderate effect on growth, while expression of mutDFNA5 triggered a significant growth defect. The latter was characterised by a longer doubling time and a lower maximal optical density of the cultures (Fig. 1b). Hence, as compared to a culture of the BY4741 strain transformed with the empty plasmid, a culture of cells expressing wtDFNA5 required on average only an additional 3 h (SD ± 0.24) to reach half of the maximal optical density (ΔT½), whereas for a culture of cells expressing mutDFNA5 this ΔT½ was extended

to about 11.5 h (SD ± 0.32) (Suppl. Data Table 2.3). Combined these data suggest that yeast cells tolerated the presence of wtDFNA5 fairly well but have problems to cope with mutDFNA5. Notably, since the mutant protein seemed to escape the protein breakdown, the cells apparently counter selected and reduced the expression of the mutant protein to prevent extreme harmful effects.

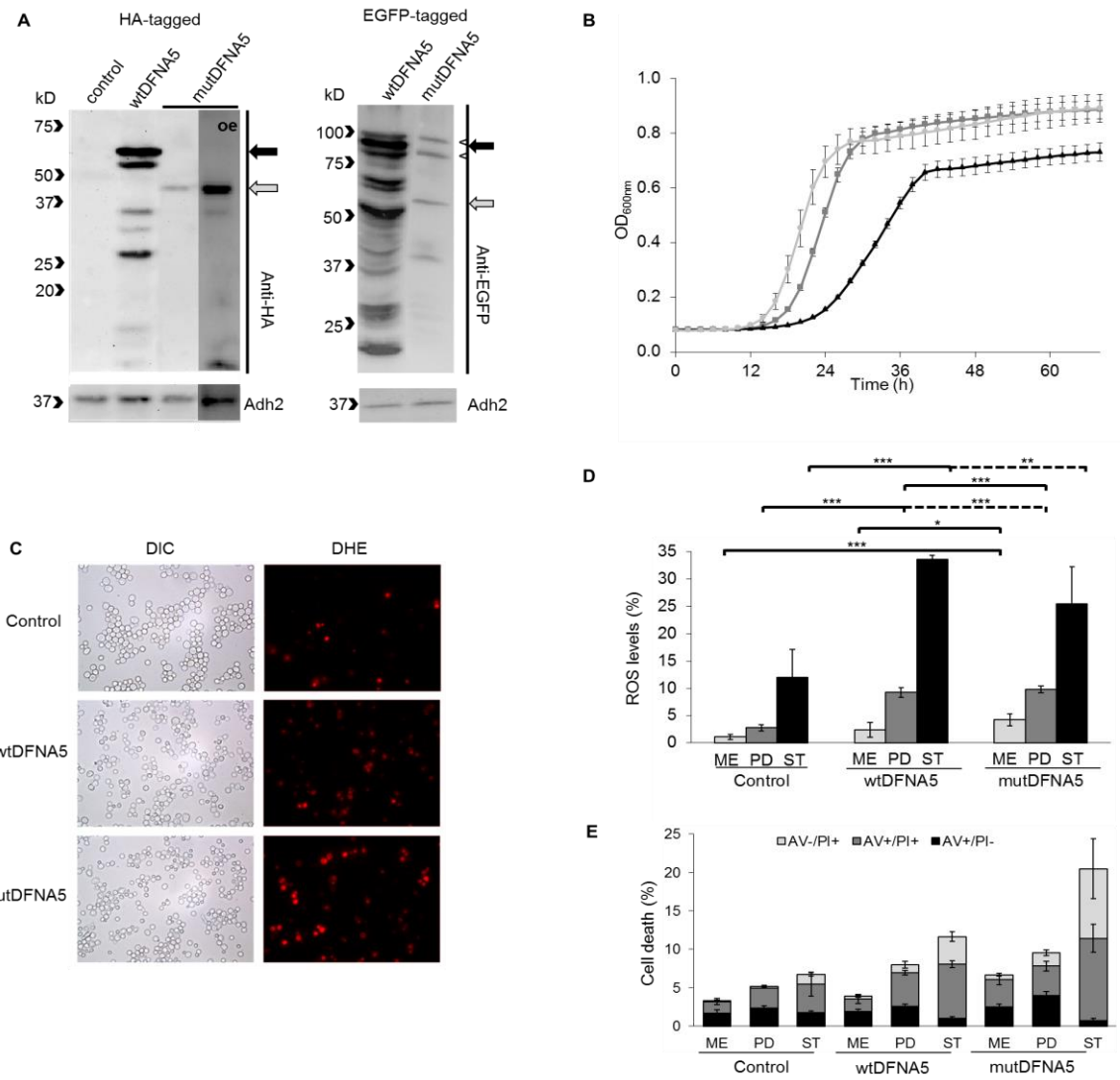

Fig. 1: Repercussions of wtDFNA5 and mutDFNA5 expression in BY4741 wild-type cells. a. Western blot analysis of protein extracts of the BY4741 wild-type strain transformed with an empty plasmid (contr.) or constructs allowing for the expression of C-terminally HA-tagged and EGFP-tagged wt*DFNA5* or mu*tDFNA5* as indicated. Immunodetection was performed using primary antibodies directed against the HA-tag, EGFP or Adh2, which was used as loading control. The black arrow indicates full-length wtDFNA5 and the grey arrow full-length mutDFNA5. The small open arrowheads indicate aspecific bands detected by anti-EGFP. For mutDFNA5, the lane marked with [oe] represents an overexposure of the western blot. **b.** Growth of the BY4741 wild-type strain transformed with an empty plasmid (light grey circles) or a construct allowing for expression of C-terminally HA-tagged wtDFNA5 (dark grey squares) or mutDFNA5 (black triangles). **c.** Visualisation of ROS producing cells in control cultures (contr.) or cultures of cells expressing C-terminally HA-tagged wtDFNA5 or mutDFNA5 in post-diauxic phase. **d.** Quantification of ROS accumulation using DHE staining in control cultures (contr.) or cultures of cells expressing C-terminally HA-tagged wtDFNA5 or mutDFNA5 when sampled at the mid-exponential growth phase (ME), at post-diauxic shift (PD) and in stationary phase (ST). **e.** Quantification of the number of AV/PI positive cells in ME, PD and ST phase of control cells or cells expressing HA-tagged wtDFNA5 or mutDFNA5. Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining the following significances were obtained when compared to the control: for wtDFNA5 in ME: AV^-/PI^+ *; for wtDFNA5 in PD: AV^+/PI^+ ***, AV^-/PI^+ *; for wtDFNA5 in ST: AV^+/PI^- *, AV^+/PI^+ *, AV^-/PI^+ **; for mutDFNA5 in ME: AV^+/PI^- *, AV^+/PI^+ **, AV^-/PI^+ **; for mutDFNA5 in PD: AV^+/PI^- **, AV^+/PI^+ *, AV^-/PI^+ ***; for mutDFNA5 in ST AV^+/PI^- **, AV^+/PI^+ **, AV^-/PI^+ *. (*=p<0.05; **=p<0.01; ***=p<0.001)

It is known that cytotoxic effects instigated by heterologous proteins are often a reflection of a failing protein quality control and clearance system, which then leads to enhanced oxidative stress and eventually increased cell death (reviewed in [11]). To examine whether this is also the case for heterologous expression of DFNA5, we measured the level of reactive oxygen species (ROS) using a DHE staining on culture samples taken at different time points during growth (Fig. 1c, d). In mid-exponential cultures, the ROS levels were only significantly increased in cells expressing mutDFNA5 when compared to the control. However, once the cultures traversed the diauxic shift and switched their metabolism to respiration, a marked increment of the ROS level was observed for both the cells expressing wtDFNA5 and those expressing mutDFNA5. In case of wtDFNA5, the level of ROS in the early post-diauxic phase was three times higher than that of the control cells and it further increased in stationary phase. With mutDFNA5, the increment in ROS in the early post-diauxic phase was comparable to wtDFNA5, though by the time these cells reached the stationary phase the average ROS level was lower than that of cells expressing wtDFNA5.

We also performed a co-staining with AV/PI to detect cells showing signs of apoptotic and necrotic cell death (Fig. 1e). This again revealed that only the expression of mutDFNA5 significantly enhanced cell death during the mid-exponential phase, while both expression of wtDFNA5 or mutDFNA5 enhanced cell death once the cultures were beyond the diauxic shift, albeit to a different extent. More in particular, we noticed that in the post-diauxic and stationary phase the expression of the wild-type and especially the mutant allele triggered an increase in the number of late apoptotic (AV^+/ PI^+) and necrotic cells (AV^-/PI^+) and that in the stationary phase this was even associated with a significant decrease in early apoptotic (AV^+/PI^-) cells. That the increments of late apoptotic and necrotic cells are most pronounced upon expression of mutDFNA5 is consistent with the observed enhanced growth defect. Furthermore, these results are also in agreement with previously reported observations of enhanced apoptotic and necrotic cell death in mammalian cells transfected with mut*DFNA5* [6, 12].

2.4.2 Mutant DFNA5 escapes protein quality control deposition and interacts with mitochondria

To analyse the intracellular localisation, we expressed wtDFNA5 and mutDFNA5 as a C-terminally tagged EGFP fusion. Their relative expression levels were comparable to those of the HA-tagged counterparts. Furthermore, similar as for the HA-tagged versions, the wtDFNA5-EGFP fusion was subject to proteolytic degradation, while this was by far less pronounced for the mutDFNA5-EGFP fusion (Fig. 1a). Despite of these similarities, we noticed that the fusion proteins induced slightly enhanced growth defects as judged from the ΔT½ values calculated based on the growth difference with control cultures expressing native EGFP (ΔT½ wtDFNA5-EGFP: 4.60h ± 1.27; ΔT½ mutDFNA5-EGFP: 15.85h ± 1.84; Suppl. Data Table 2.3). Nonetheless, since also in this case mutDFNA5-EGFP instigated a much higher cytotoxicity than wtDFNA5-EGFP, we reasoned that further analysis would still provide important insight in the differential properties of the proteins.

Fluorescence microscopy confirmed the difference in expression level between wtDFNA5-EGFP and mutDFNA5-EGFP. It also showed that wtDFNA5-EGFP was evenly distributed over the cytoplasm in mid-exponential growth phase, although we noticed that one fifth of the cells (22%) gradually accumulated fluorescent material in more dense inclusions (Fig. 2a). This resulted in the formation of one or a few distinct deposits by the time these cells reached the post-diauxic phase. Similar as in transfected mammalian cells [12], the distribution of mutDFNA5-EGFP in mid-exponential phase cells appeared to be more granulated and possibly confined to intracellular structures, though it was difficult to assign a definite pattern due to the low expression level of the fusion protein. In the post-diauxic phase, inclusions were present in about one out of seven cells that expressed mutDFNA5-EGFP (14%). As compared to the deposits of wtDFNA5-EGFP, the inclusions formed by the mutant protein were usually less intense and occurring as small foci in the vicinity of the plasma membrane (Fig. 2a).

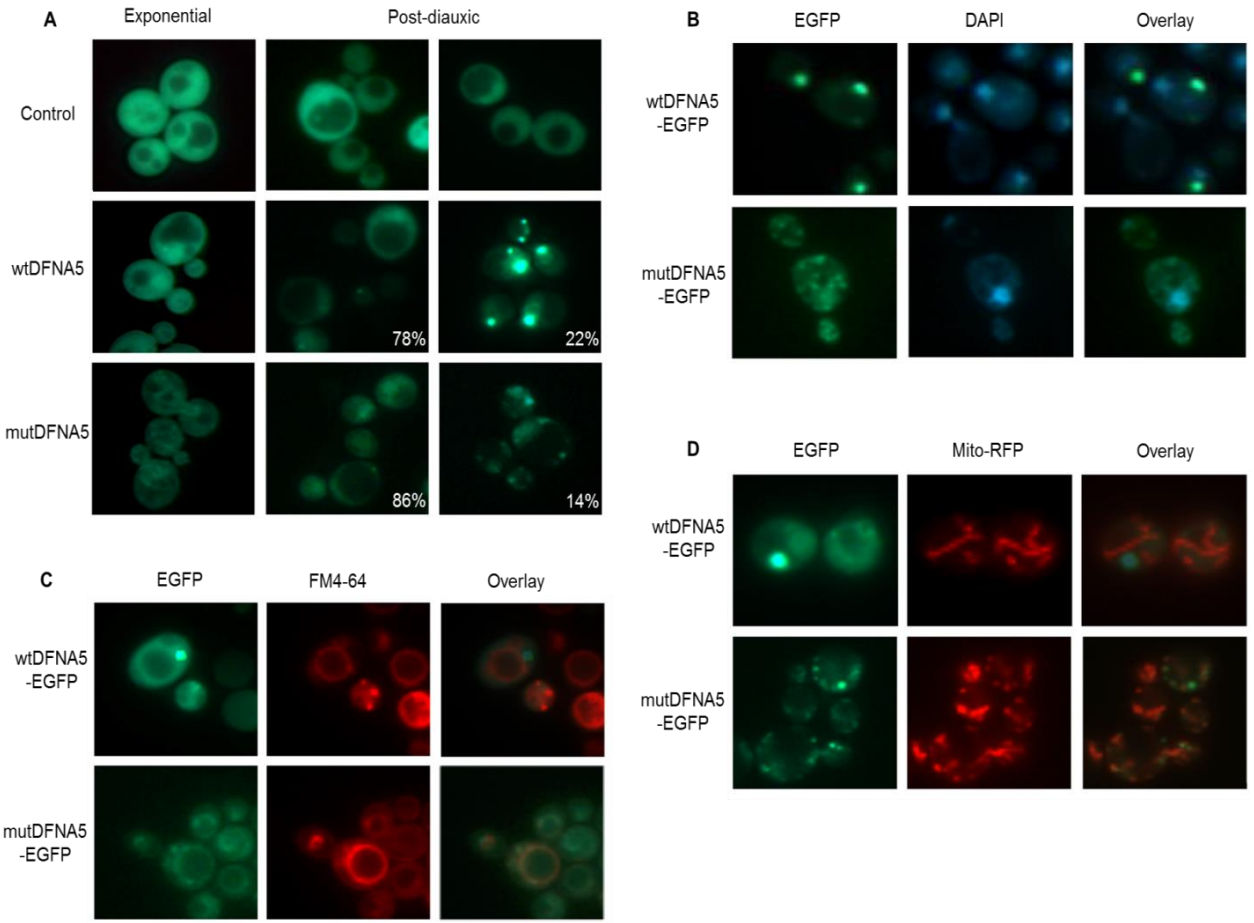

Fig. 2: Wild-type and mutant DFNA5 form inclusions in yeast. a. Fluorescence microscopic visualisation and intracellular localisation of wtDFNA5-EGFP and mutDFNA5-EGFP fusion proteins in the BY4741 wild-type yeast strain sampled at the mid-exponential growth phase or in the post-diauxic growth phase as indicated. Percentages refer to the number of cells displaying a dispersed cytoplasmic localisation or with inclusions. Cells expressing only EGFP served as control. **b, c.** Pictures of post-diauxic wild-type cells with inclusions formed by wtDFNA5-EGFP or mutDFNA5-EGFP and stained with DAPI (panel B) to visualise the nucleus or with FM4-64 (panel C) to visualise the vacuolar membrane. **d.** Pictures of post-diauxic wild-type cells expressing wtDFNA5-EGFP or mutDFNA5-EGFP and a mitochondrial red fluorescent marker protein, Mito-RFP.

It was previously shown that cells protect themselves by sequestering breakdown products and damaged or aggregated proteins in different protein quality control compartments, referred to as aggresomes or JUNQ and IPOD. JUNQ represents a juxta-nuclear quality control compartment that serves as a temporary storage site for misfolded proteins, keeping them in an ubiquitinated, soluble state for either refolding or degradation by the ubiquitin-proteasome system. IPOD, on the other hand, is a perivacuolar compartment for the deposit of insoluble, non-ubiquitinated substrates, such as amyloidic proteins, that possibly await clearance by means of autophagy [13, 14]. To analyse in more detail the localisation of the inclusions formed by wtDFNA5-EGFP and mutDFNA5-EGFP, we performed stainings with DAPI and FM4-64 to respectively visualise the nucleus and the vacuolar membrane. This demonstrated that the larger deposits of wtDFNA5-EGFP did not co-localise with the nucleus (Fig. 2b). Instead, these deposits were found at the periphery of the vacuole and thus are likely to correspond to IPOD (Fig. 2c). The small foci formed by mutDFNA5-EGFP, neither co-localised with the nucleus, nor with the vacuolar membrane but, interestingly, seemed to partially overlap with DAPI-stained mitochondrial DNA. This led us to visualise the mitochondrial network. The strains were therefore co-transformed with a plasmid enabling the expression of a mitochondrial red fluorescent marker protein, Mito-RFP [7]. Further analysis revealed that, indeed, the small foci of mutDFNA5-EGFP often co-localised with punctuated fragmented mitochondria and this in contrast to the larger deposits of wtDFNA5-EGFP (Fig. 2d).

2.4.3 Mutant DFNA5 induces cell death independently of caspase

Next, we systematically assessed the repercussion of wtDFNA5 and mutDFNA5 expression in strains harbouring deletions of key players of the PCD machinery. We monitored the expression of the HA-tagged DFNA5 proteins, compared the growth profiles and measured the levels of ROS and cell death during mid-exponential growth. One of the strains analysed was the mutant lacking the yeast caspase, Mca1, which allowed us to establish whether DFNA5-induced cell death involves the previously described caspase-dependent or caspase-independent processes [15]. As compared to the corresponding wild-type strains, the mca1Δ strains displayed similar expression profiles of wtDFNA5 and mutDFNA5 (Fig. 3a) and albeit the mutant strains grew more slowly, they maintained similar DFNA5-dependent growth defects as determined by calculating the ΔT½ values (Suppl. Table 2.3 and Fig. 3b). In addition, the deletion of MCA1 did not prevent accumulation of ROS upon expression of mutDFNA5, nor did it prevent the mutDFNA5-instigated induction of apoptotic and necrotic cell death markers (Fig. 3c, d). Consistently, treatment of BY4741 wild-type cells with the FITC-labelled pancaspase inhibitor zVAD-FMK, which binds to the active site of caspases in yeast [10], did not provide evidence for enhanced caspase activity upon the expression of wtDFNA5 or mutDFNA5 (Fig. 3e). When combined, these observations suggest that mutDFNA5-induced cell death occurs mainly independent of the caspase Mca1. Likewise, we could exclude several of the other known players of the yeast apoptotic machinery to have a major impact on the DFNA5-dependent phenotypes. These included the mitochondrial cell death effector, Aif1, the mitochondrial endonuclease G, Nuc1, the orthologue of the mammalian Omi/HtrA2 serine protease, Nma111, as well as the yeast suicide protein, Ysp2 (Suppl. Data Table 2.3 and data not shown).

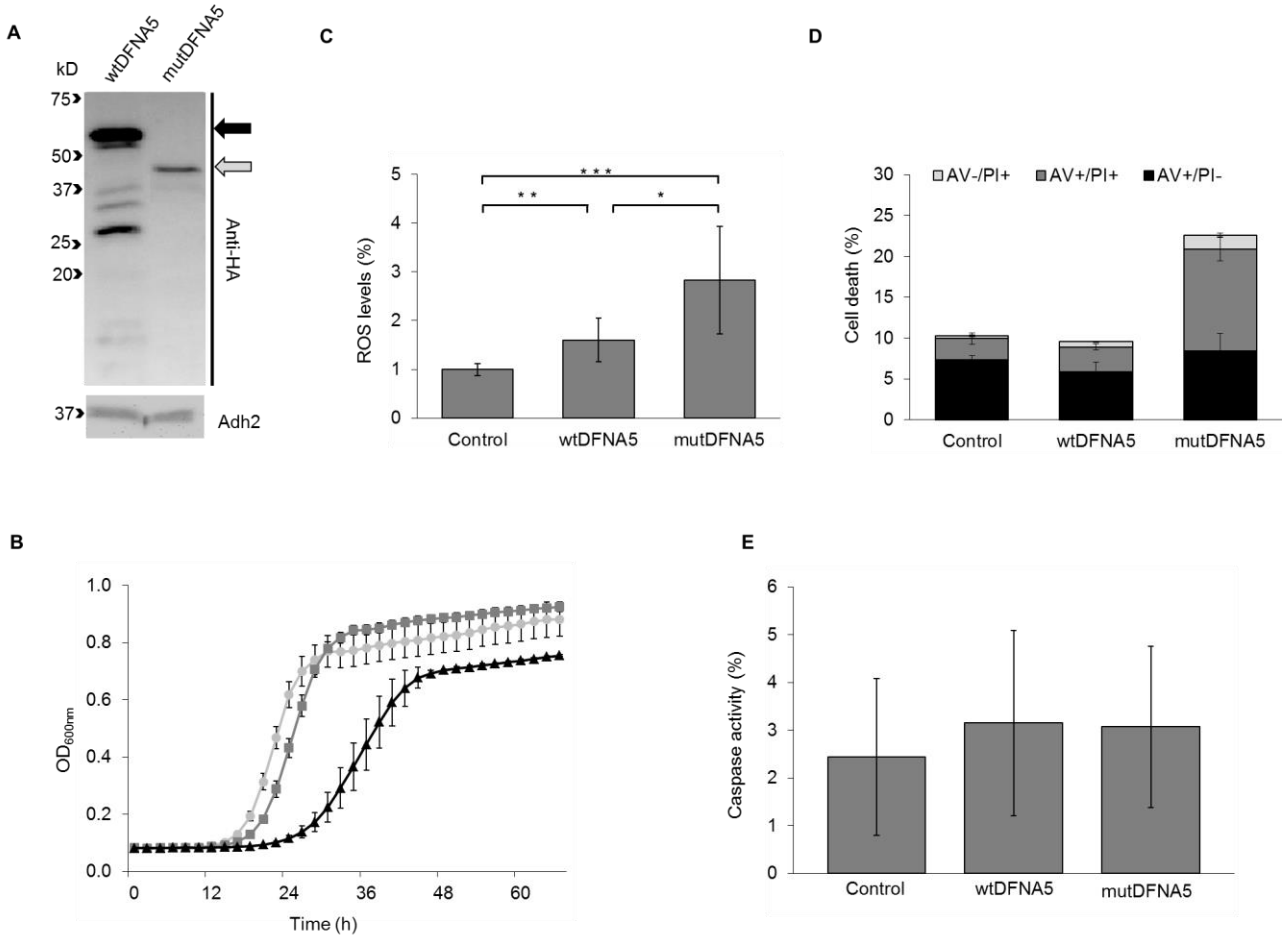

Fig. 3: MutDFNA5-induced cell death does not involve the yeast caspase. a. Western blot analysis of protein extracts of *mca1Δ* cells transformed with constructs allowing for the expression of C-terminally HA-tagged wtDFNA5 or mutDFNA5 as indicated. Immunodetection was performed using primary antibodies directed against the HA-tag or Adh2, which was used as loading control. The black arrow indicates full-length wtDFNA5 and the grey arrow full-length mutDFNA5. **b.** Growth profiles of *mca1Δ* cells expressing HA-tagged wtDFNA5 (dark grey squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light grey circles). **c, d.** Quantification of ROS accumulation using DHE staining (panel C) or AV/PI positive cells (panel D) in mid-exponential cultures of *mca1Δ* cells transformed with an empty plasmid (contr.) or with constructs allowing for expression of C-terminally HA-tagged wtDFNA5 or mutDFNA5. **e.** Assessment of the caspase activity using FITC-tagged z-VAD-FMK in BY4741 wild-type cells expressing C-terminally HA-tagged wtDFNA5 or mutDFNA5 or transformed with an empty plasmid (contr.). Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining the following significances were obtained when making the comparison with the control: wtDFNA5: AV^-/PI^+ *; mutDFNA5: AV^+/PI^+ ***, AV^-/PI^+ ***. (*=p<0.05; **=p<0.01; ***=p<0.001)

2.4.4 Mutant DFNA5 induces cell death through mitochondrial functions

In contrast to the mutant strains mentioned above, we found increased DFNA5-induced cytotoxicity in the strains lacking either the mitochondrial outer membrane protein Fis1, or the voltage-dependent anion channel protein Por1. The Fis1 protein is involved in mitochondrial fission that attracts the dynamin-related GTPase, Dnm1 through the adaptors Mdv1 and Caf4. The complex then forms a contractile ring that promotes outer membrane division. Interesting is that with the *fis1Δ* strains, both the cultures expressing wtDFNA5 or mutDFNA5 displayed a comparable lower maximal optical density (Fig. 4a). This is similar to what we observed for cultures of wild-type cells transformed with mut*DFNA5* and it probably reflects the disturbance of mitochondrial dynamics. The role of Fis1 and mitochondrial fission in PCD is still not fully clarified and seems to depend on the type of the cell death stimulus [16]. For instance, for ethanol-induced apoptosis, Fis1 was shown to mediate mitochondrial fragmentation and cell death independently of Dnm1 and Mdv1 [17], whereas for acetic acid-induced apoptosis, Fis1 was reported to protect cells by inhibition of Dnm1- and Mdv1-mediated mitochondrial fission and cell death [18]. Concerning the DFNA5-induced cell death, Fis1 obviously exerted a protective function, but this appeared to be largely independent of Dnm1 and Mdv1 because neither the deletion of *Dnm1*, nor the deletion of *Mdv1* affected the DFNA5-mediated growth phenotype (Suppl. Data Table 2.3). As was to be expected, the levels of ROS were generally higher in the *fis1Δ* strains than in the wild-type strains with a minor increment in case of wtDFNA5 expression, but a clear augmentation in case of mutDFNA5 expression (Fig. 4b). Likewise, the amount of dying cells in mid-exponential cultures were in general higher in the *fis1Δ* strains as compared to the wild-type strains, especially the number of early apoptotic cells, and while there was no significant increase in the total number of cells showing signs of cell death between the control and cultures expressing wtDFNA5 or mutDFNA5, the latter two still showed a trend towards enhanced late apoptosis and necrosis (Fig. 4c). Analysis of *fis1Δ* cells with combined expression of Mito-RFP and EGFP-fusions showed that most cells contained fragmented and aggregated mitochondria, which did not overlap with the deposits formed by wtDFNA5-EGFP but clearly co-

localised with the foci of mutDFNA5-EGFP (Fig. 4d). Similar as the loss of Fis1, also the absence of the channel protein Por1 appeared to sensitise cells for DFNA5-mediated cell death. However, with this *por1Δ* deletion strain it was difficult to correctly assess the repercussions on ROS accumulation or cell death due to the very slow growth of the cells transformed with mutDFNA5 (Fig. 4e).

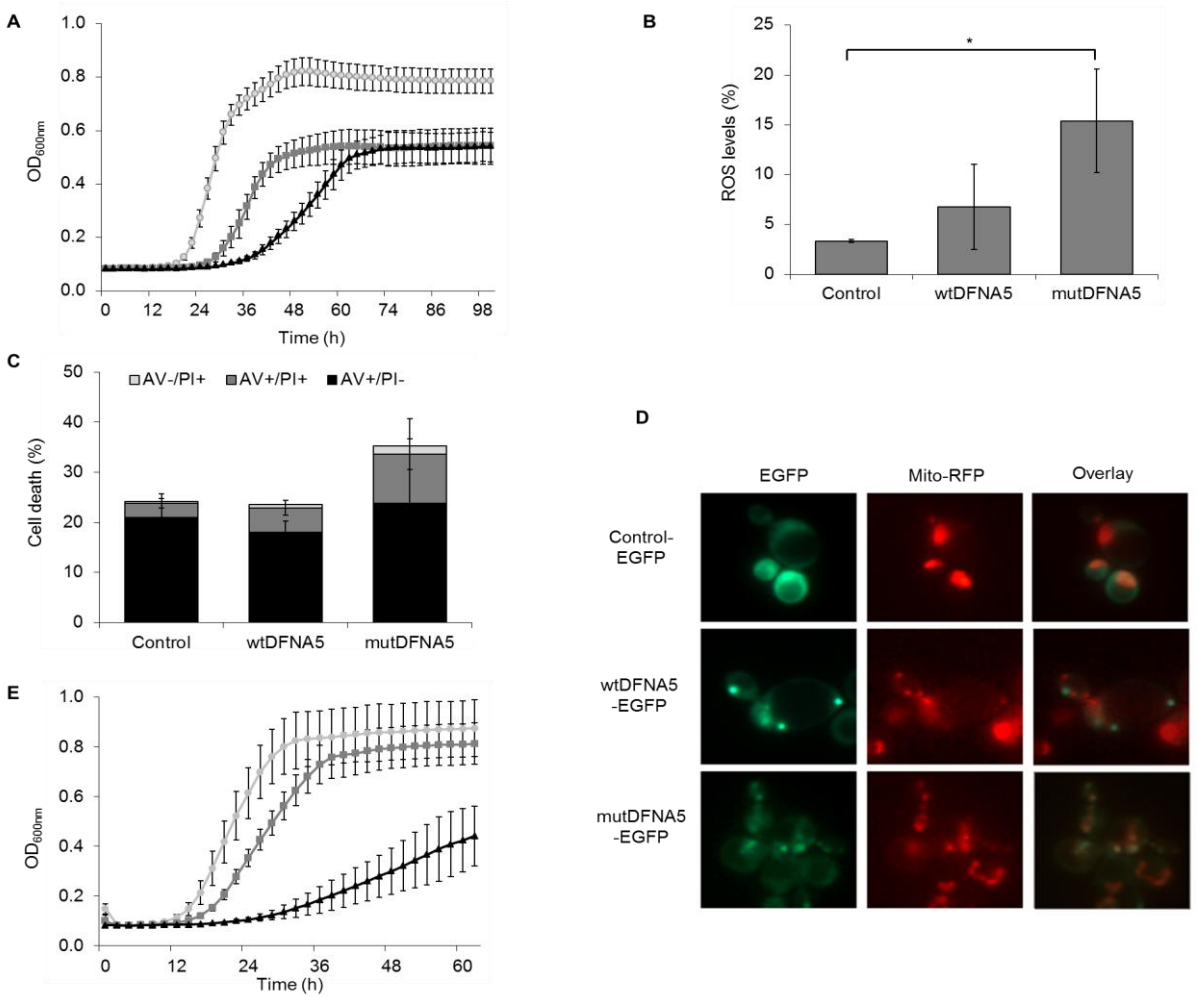

Fig. 4: DFNA5 cytotoxicity is enhanced in cells lacking mitochondrial functions. a. Growth profiles of *fis1Δ* cells expressing C-terminally HA-tagged wtDFNA5 (dark grey squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light grey circles). **b, c.** Quantification of ROS accumulation using DHE staining (panel B) or AV/PI positive cells (panel C) in mid-exponential *fis1Δ* cultures without (contr.) or with expression of HA-tagged wtDFNA5 or mutDFNA5. **d.** Fluorescence microscopy pictures of *fis1Δ* cells expressing EGFP or displaying inclusions of wtDFNA5-EGFP or mutDFNA5-EGFP and co-transformed with Mito-RFP to visualise mitochondria. **e.** Growth profiles of *por1Δ* cells expressing HA-tagged wtDFNA5 (dark grey squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light grey circles). Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining of the *fis1Δ* cultures the following significant p-values were obtained when making the comparison with the control and mutDFNA5: AV^+ / PI^+ **. (*=p<0.05; **=p<0.001).

In two other mitochondrial mutants, *i.e.* the *aac1Δ* and *aac3Δ* deletion strains (Fig. 5a, b), the growth differences between the control cultures and the cultures expressing wtDFNA5 or mutDFNA5 were almost annihilated, indicative that the lack of Aac1 or Aac3 abrogated the mutDFNA5-associated cytotoxicity. *Aac1* and *Aac3* encode for two of the three ADP/ATP carriers of the inner mitochondrial membrane. Previously reported studies implicated these proteins as effectors of acetic acid-induced apoptosis, a role which apparently does not depend on their ADP/ATP translocase activity but rather on their impact on the mitochondrial outer membrane permeabilisation and mitochondrial degradation [19, 20]. As documented for the *aac3Δ* mutant, the deletion of the ADP/ATP carrier did not prevent the accumulation of ROS during the post-diauxic phase in cells expressing wtDFNA5 or mutDFNA5 (Fig. 5c). However, the lack of Aac3 clearly interfered with the appearance of cell death markers. In the *aac3Δ* control cultures, the levels of dying cells were markedly higher as compared to control culture of wild-type cells, which is consistent with the fact that the *aac3Δ* mutant grows slower. The cultures with cells expressing wtDFNA5 or mutDFNA5 still displayed altered ratios between early or late apoptotic and necrotic cells, but the total number of cells with signs of cell death did not alter in the different growth phases. Furthermore, the total number of dying cells was comparable for the control culture and the culture of cells expressing wtDFNA5 and it was consistently lower for the culture of cells expressing mutDFNA5 (Fig. 5d). Similar data were obtained for the *aac1Δ* mutant (data not shown). These observations confirm that mutDFNA5 requires the ADP/ATP carriers to instigate cytotoxicity and cell death. Furthermore, while ROS production has been described as an event common to most of the yeast apoptosis scenarios, our data demonstrate that in the ADP/ATP carrier mutants the correlation between ROS accumulation and viability does not hold. As such, our data are completely in line with previously reported results obtained with a triple *aac1-3Δ* mutant for acetic acid-induced apoptosis [19].

Analysis of *aac3Δ* cells co-transformed with Mito-RFP and the EGFP-fusions revealed that these cells contain a well-developed mitochondrial tubular

network. Even in cells expressing mutDFNA5-EGFP such a tubular network was present, but there were still punctuated mitochondria co-localising with the foci of the EGFP fusion (Fig. 5e). This led us to conclude that the absence of the ADP/ATP carriers did not prevent mutDFNA5 to interfere with mitochondrial fission and fusion dynamics or the clearance of fragmented mitochondria, which both are aspects that remain to be studied in more detail.

Finally, it should be noted that we did not observe significant differences in expression or degradation of the HA-tagged wtDFNA5 or mutDFNA5 proteins between the wild-type strain and the different mutant strains (Fig. 5f). This indicates that the observed changes in DFNA5-instigated cytotoxicity in the mutant strains are related to their deleted functions and not to alterations in DFNA5 expression.

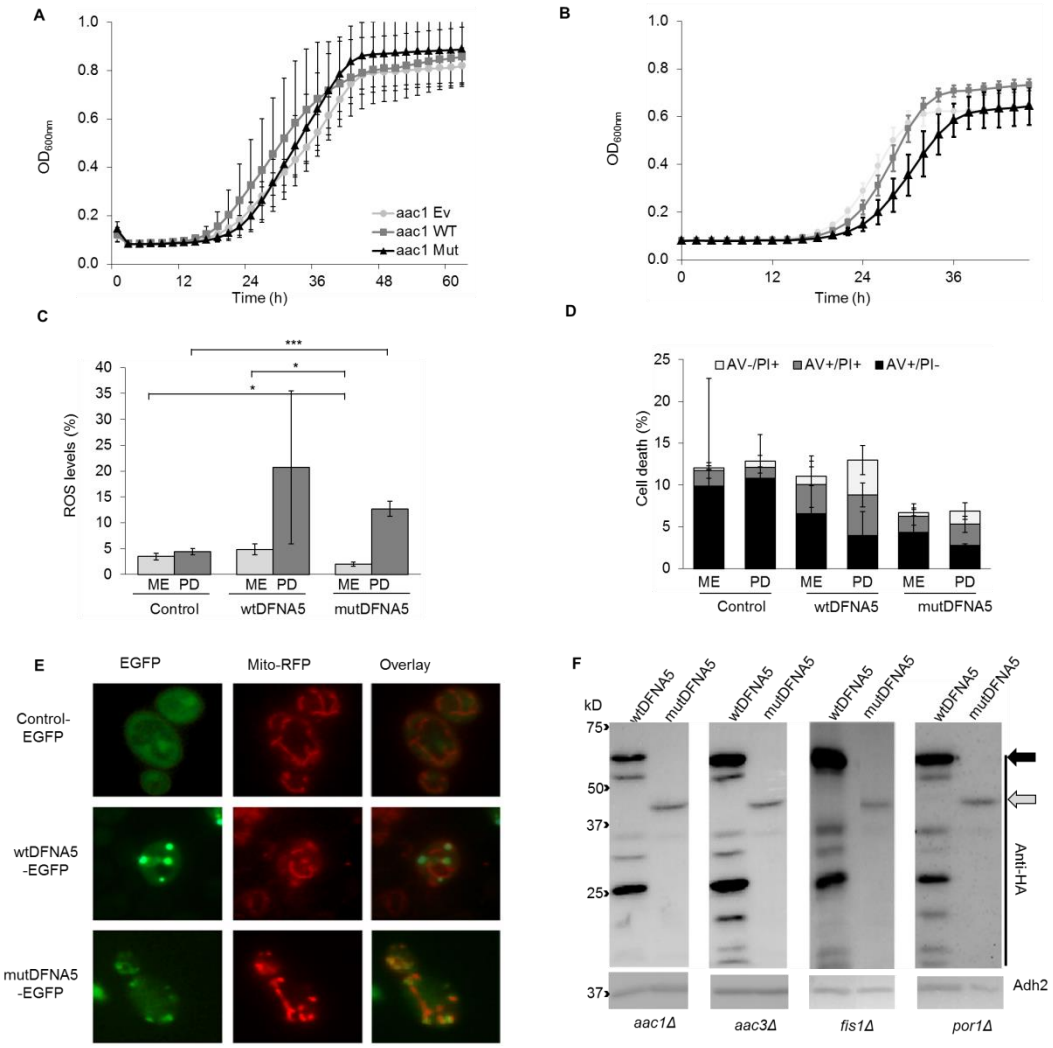

Fig. 5: MutDFNA5 cytotoxicity depends on the mitochondrial ADP/ATP carriers. a, b. Growth profiles of (A) *aac1Δ* cells or (B) *aac3Δ* cells expressing C-terminally HA-tagged wtDFNA5 (dark grey squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light grey circles). **c, d.** Quantification of ROS accumulation using DHE staining (panel C) or AV/PI positive cells (panel D) in mid-exponential (ME) and post-diauxic (PD) phase in *aac3Δ* cultures without (contr.) or with expression of HA-tagged wtDFNA5 or mutDFNA5. **e.** Fluorescence microscopy pictures of *aac3Δ* cells expressing EGFP or displaying inclusions of wtDFNA5-EGFP or mutDFNA5-EGFP and co-transformed with Mito-RFP to visualise mitochondria. **f.** Western blot analysis of protein extracts of the *aac1Δ*, *aac3Δ, fis1Δ, por1Δ* mutant strains transformed with constructs allowing for the expression of C-terminally HA-tagged wtDFNA5 or mutDFNA5 as indicated. Immunodetection was performed using primary antibodies directed against the HA-tag or Adh2, which was used as loading control. Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining the following significances were obtained when compared to the control: for wtDFNA5 in PD: AV^+/PI^+ *, AV^-/PI^+ *. (*=p<0.05; ***=p<0.001)

2.4.5 DFNA5 toxicity is confined to its first globular domain

We recently proposed that wtDFNA5 is composed of two globular domains, which are separated by a hinge region (Fig. 6a). In that study, we also demonstrated that the first domain induces apoptotic cell death in transfected HEK293T cells, which led to a model where the second domain can fold back to mask and regulate the apoptotic activity of the first domain [6]. Here, we expressed the two domains separately in yeast. As shown, the expression of the first domain, designated domain A and corresponding to the amino acid residues 1 to 256, triggered a very pronounced growth defect that by far surpassed the defect observed for mutDFNA5 (Fig. 6b). Expression of the second domain, referred to as domain B and corresponding to residues 282 to 496, did not affect growth and the growth curve almost perfectly overlapped with the one obtained for the empty vector control.

DFNA5 belongs to the gasdermin protein family, named after the founder protein GSDMA, which is involved in gastric cancer and also contains pro-apoptotic activities [21]. Sequence alignment of the different gasdermin family members show a high degree of conservation, especially in the first domain, which based on hydrophobic cluster analysis contains short α-helical folds interspaced by β-sheets [6]. To document the importance of these structures for the apoptotic-inducing activity of the domain, we used PCR-based site-directed mutagenesis and phenotypically tested two of the mutants generated in domain A (Fig. 6a). The first mutant, designated HCA-F2R, contained the substitution of a highly conserved hydrophobic phenylalanine into a basic arginine, thereby disrupting the first α-helical fold. When expressed in yeast, this mutant only triggered a small growth defect (ΔT½: 3.33h ± 0.93) and thus lost most of the apoptotic-inducing activity (Fig. 6B). In the second mutant, *i.e.* HCA-A3R, a non-conserved alanine residue was changed into arginine. Although this mutation also affected the first α-helical fold, its repercussions on the apoptotic-inducing activity were less pronounced as evidenced by the observation that the expression of this mutant in yeast resulted in an intermediate growth defect (ΔT½: 20.50h ± 5.96; Fig. 6b).

Next to the experiments in yeast, we also assessed the cell viability of human HEK293T cells transiently expressing the generated DFNA5 mutants. The expression of the HCA-A3R construct led to a significant decrease of cell viability (mean viability: 38.28% ± 9.37), whereas expression of HCA-F2R did not (mean viability: 78.60% ± 4.03), as it gave a similar cell viability as that observed for cells transfected with an empty EGFP vector (mean viability: 77.59% ± 5.58; Fig. 6c). In fact, the cell viability of cells expressing HCA-A3R is highly comparable to those expressing mutDFNA5, while cell viability of cells expressing HCA-F2R is comparable to cells expressing wtDFNA5. As such, the results obtained in HEK293T cells confirm those obtained in yeast.

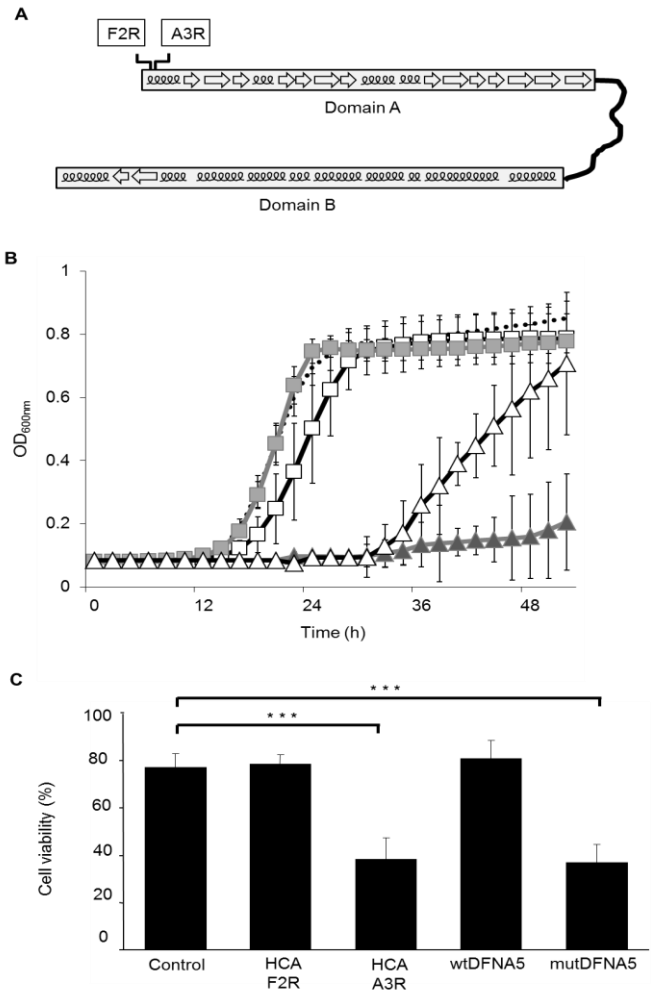

Fig.6: DFNA5 cytotoxicity is confined to its first globular domain. a. Schematic representation of wtDFNA5 with the two globular domains connected by the hinge region and the indication of the mutants HCA-F2R and HCA-A3R (boxed) in the first domain. **b.** Growth profiles of BY4741 cells expressing the first (grey triangles) or the second (greys squares) globular domain of wtDFNA5 or expressing point mutants in the first globular domain, i.e. mutants HCA-F2R (open squares) or HCA-A3R (open triangles). The dashed line is given as reference and depicts the growth of BY4741 cells transformed with an empty plasmid. **c.** Cell viability of HEK293T cells transiently expressing the HCA-F2R and HCA-A3R mutants, wtDFNA5 and mutDFNA5 as indicated. Significances were assayed using One-Way ANOVA (***= $p < 0.001$). Cells expressing EGFP served as control.

2.5 Discussion

In this study we analysed the repercussion of heterologous expression of human wtDFNA5 or mutDFNA5 in *Saccharomyces cerevisiae*. Our data clearly demonstrate that mutDFNA5 causes a significant growth defect, which is associated with an increased number of late apoptotic and necrotic cells especially when the culture entered the stationary phase and this in contrast to wtDFNA5. These findings confirm previous reported results showing that mutDFNA5 induces apoptotic and necrotic cell death when expressed in mammalian cells [6,12].

Detailed analysis of the expression of wtDFNA5 and mutDFNA5 in the yeast system revealed that both proteins form inclusions. For wtDFNA5, these inclusions most likely correspond to IPOD as they are found at the periphery of the vacuole. The IPOD is a protein quality control compartment where proteins are deposited that presumably await autophagic clearance [13,14]. This suggests that wtDFNA5 is subjected to the normal cellular repertoire of protein quality control and clearance mechanisms. MutDFNA5 seems to escape these quality control systems. It does not form large IPOD-like deposits but rather smaller and more numerous foci, which are less intense and occurring mostly in the vicinity of the plasma membrane. Previous studies already suggested an association of mutDFNA5 with the plasma membrane and/or a membrane protein in mammalian cells, but the exact location remained unclear [12,22]. We now show that in yeast the foci formed by mutDFNA5 often co-localise with fragmented mitochondria, suggesting that the mutant proteins may interact either with the mitochondrial membrane or one of the mitochondrial membrane proteins, which in turn may lead to mitochondrial impairment. The latter is further evidenced by the fact that mutDFNA5 is even more toxic in the *fis1Δ* and *por1Δ* mutants, while it lost its specific toxicity-inducing capacity in mutant strains lacking the ADP/ATP carriers Aac1 and Aac3. It is remarkable that the same mitochondrial proteins have previously been identified as key players with similar contributions for acetic acid-induced apoptosis in yeast [18,19]. This underscores that mutDFNA5-instigated cytotoxicity and acetic acid-induced apoptosis build on common molecular mechanisms. Note that we

did not analyse the third ADP/ATP carrier Aac2 in our studies for the simple reason that its deletion is lethal in the BY4741 background [23].

The studies on acetic-acid-induced apoptosis demonstrated a protective function of Fis1 that relates to inhibition of Dnm1-mediated mitochondrial fission and possible additional pro-apoptotic Dnm1 functions [18]. Also for mutDFNA5-induced cell death we found that Fis1 fulfils a protective role but this apparently does not involve Dnm1 or its adaptor Mdv1. Indeed, our observation that neither the deletion of *DNM1* nor of *MDV1* prevented mutDFNA5-induced cell death excludes these fission proteins as downstream effectors. That Fis1 could have a specific function not shared by the other fission factors Dnm1 and Mdv1 was already noted before in studies dealing with ethanol-induced apoptosis. These studies suggested that Fis1 has a specific role for the maintenance of mitochondrial fragmentation in response to ethanol [17]. However, most recent studies revealed that *fis1Δ* mutants accumulate secondary loss-of-function mutations in the *WHI2* gene [24, 25], which encodes a protein involved in cell cycle regulation [26], the general stress response, actin dynamics and Ras-cAMP-PKA signalling [27, 28] as well as the selective degradation of dysfunctional mitochondria via autophagy, a process known as mitophagy [29]. In fact, the studies on cell death and mitophagy showed that the enhanced sensitivity of the *fis1Δ* mutants towards cell death stimuli is solely due to the loss of the Whi2 function and not to the lack of Fis1 [24, 25]. Also the *fis1Δ* mutant strain of the yeast deletion collection that was used in our study appears to contain such a secondary loss-of-function mutation in *WHI2* [24]. Hence, it is feasible that the enhanced mutDFNA5-instigated cytotoxicity in the *fis1Δ* mutant strain relates to the *WHI2* mutation and the consequent diminished stress resistance and lower rate of mitophagy, rather than to a deficiency in mitochondrial fission. At least, it would explain the observed accumulation of fragmented and aggregated mitochondria co-localising with the foci of mutDFNA5-EGFP in the *fis1Δ* mutant, and as such provide an additional confirmation that mutDFNA5 triggers cell death through mitochondrial damage.

Another aspect of Fis1 is that the protein has similar cellular properties as the mammalian BCL2 and BCL-xL and although these anti-apoptotic proteins cannot replace the mitochondrial fission function of Fis1, they do substitute for Fis1 in cell viability assays [18]. BCL2 and BCL-xL have important roles as regulators of mitochondrial membrane permeabilisation, since they inhibit non-specific pore formation by the adenine nucleotide translocator, ANT, the mammalian orthologue of the Aac1/2/3 ADP/ATP carriers [30, 31]. Previous studies in yeast identified Fis1 as a potential regulator together with the mitochondrial permeability transition pore components Aac1/3 and the VDAC protein Por1 for acetic acid-induced cell death [18-20]. Our data now demonstrate that mutDFNA5 cytotoxicity is enhanced in the absence of Por1, while this toxicity is basically abrogated in the absence of Aac1 or Aac3. Whether this means that mutDFNA5 directly targets the ADP/ATP carriers to alter mitochondrial membrane permeability remains to be clarified. In humans, mutations in the *ANT1* gene are associated with progressive external ophthalmoplegia [32] and to our knowledge there are no reports that link *DFNA5* to this disorder or, conversely, that link *ANT1* to autosomal dominant deafness.

DFNA5 belongs to the gasdermin family. Although the members of this family appear to have different molecular functions, they share conserved structural features, such as the presence of a globular domain in their N-terminal half [6, 21, 33]. Intriguingly, this domain harbors the DFNA5 capacity to induce cell death as confirmed in previous studies [6] and ours. It is not known whether this capacity to induce cell death is a common physiological property of all gasdermin family members, but at least for one other member, *i.e.* GSDMA, this seems to be the case since the protein was reported to induce apoptosis in a gastric epithelial cell line [21]. The apoptosis-inducing globular domain was proposed to be shielded in wtDFNA5 by a second C-terminal regulatory domain. In mutDFNA5 a large part of this regulatory domain is missing and therefore the apoptosis-inducing domain is presumably more exposed [6, 22]. Structurally, the apoptosis-inducing domain is composed of short α-helical folds interspaced by β-sheets. Here, we show that mutations disrupting the first α-helical fold

strongly reduce the cell death-inducing capacity of the N-terminal domain in yeast and human HEK293T cells. This demonstrates the feasibility to use the yeast system to further dissect the structural requirements of DFNA5 associated with its apoptosis-inducing property.

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Chapter III

Mutant DFNA5 induces cell death by mitochondria and MAPK-related pathways

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Modified from a submitted manuscript

All the DFNA5 plasmid constructs used in this chapter were made by Dr. Ken Op de Beeck. The microarray experiment in yeast was performed at the 'VIB Nucleomics Core' where the RNA underwent a quality control, reversed transcription, labelling and hybridisation. The bioinformatics analysis for the Agilent array in R was performed in cooperation with Vesna Hristovska. The microarray experiment in human cell lines and the subsequent bioinformatics analysis for the Illumina array was performed by Dr. Ken Op de Beeck. Subsequent GO analysis and the rest of the scientific experiments were performed by Sofie Van Rossom. All the co-authors contributed to the corrections and revision of the written report.

3 Mutant DFNA5 induces cell death by mitochondria and MAPK-related pathways

3.1 Abstract

Cell death exists in many different forms. Some are accidental, but most of them encounter some kind of regulation and are called programmed cell death. This study investigated programmed cell death induced by *DFNA5*, a gene responsible for autosomal dominant hearing loss and a tumour suppressor gene involved in frequent forms of cancer. Recently, functional analysis of mutant DFNA5 linked expression to programmed cell death both in human cell lines and in *Saccharomyces cerevisiae*. To further investigate the cell death mechanism induced by mutant DFNA5, we performed a microarray study in both models using respectively an Illumina human HT12v3 bead chip and an Agilent Quick Amp 2 colour 8x15K slide.

When using wild-type *DFNA5* as reference, which in contrast to mutant DFNA5 does not induce cell death, our data show that the yeast pathways related to mitochondrial ATP-coupled electron transport chain, oxidative phosphorylation and energy metabolism are up-regulated, while in human cell lines, MAP kinase-related activity is up-regulated. Inhibition of this pathway is able to partially attenuate the resulting cell death induced by mutant DFNA5 in human cell lines. In yeast, the association with the mitochondria was demonstrated by the up-regulation of several cytochrome c oxidase genes involved in the cellular oxidative stress production. The link between the mitochondria and the MAPK pathway is however unknown at this moment but could be mediated by oxidative stress.

Both models show a down-regulation of protein sorting- and folding-related mechanisms (related to the endoplasmic reticulum (ER) in yeast) which could indicate a role for the ER. The exact relationship between the ER and the mitochondria in DFNA5-induced cell death remains unknown at this moment, but these results suggest a potential relation between those organelles, triggering a proper cellular response in mutDFNA5-induced cell death. Further elucidation of the DFNA5-related cell death mechanism can

lead to new insights and therapies related to HL and may yield possible therapeutic targets for DFNA5-induced HL.

3.2 Introduction

DFNA5 was originally identified as a gene responsible for an autosomal dominant form of hearing loss (HL) in a Dutch family [1]. In addition to HL, *DFNA5* has also been correlated with several forms of cancer, such as breast, colorectal, hepatocellular and gastric cancer. Endogenous *DFNA5* is epigenetically silenced by hypermethylation in cancer cells resulting in a decreased *DFNA5* expression level [2-5].

Recently, functional studies in human cell lines revealed that DFNA5 is correlated with programmed cell death (PCD). Human HEK293T cells were used to study the function of DFNA5 and transfection of mutant *DFNA5* resulted in an apoptotic growth defect compared to wild-type *DFNA5* [6, 7]. Additionally, experiments in fission yeast revealed a cell cycle arrest upon expression of mut*DFNA5* [8]. In chapter II we demonstrated the presence of this growth defect in the budding yeast *Saccharomyces cerevisiae* confirming the value of this model organism to unravel the mechanisms related to DFNA5. Moreover, we also demonstrated a role of mitochondria in *DFNA5*-related cell death in *Saccharomyces cerevisiae* (described in chapter two). Several mitochondrial proteins, such as Fis1, Por1, Aac1 and Aac3, were identified to be involved in mut*DFNA5*-induced cellular toxicity. All these proteins are functionally related to mitochondrial fission or permeabilisation. Moreover, mutDFNA5 was found to co-localise with a mitochondria-targeted red fluorescent protein, Mito-RFP [9]. These former observations confirmed the role of mitochondria in cell death mechanisms in yeast, more in particular for *DFNA5*-related processes. Due to the high degree of conservation of cell death mechanisms between yeast and human, we decided to further investigate DFNA5-related mechanisms both in *Saccharomyces cerevisiae* and in human HEK293T cells.

In order to further elucidate cell death-related to DFNA5, a transcriptomic analysis was performed in *Saccharomyces cerevisiae* and in human HEK293T

cells transfected with either wild-type *DFNA5* (wt*DFNA5*) or mutant *DFNA5* (mut*DFNA5*). In the current study, we confirmed the significance of the mitochondria, more specific the cellular ATP-related processes and oxidative phosphorylation, in *DFNA5*-induced cell death in *Saccharomyces cerevisiae*. Additionally, a potential role could also be assigned to the endoplasmic reticulum (ER) as a gene ontology analysis identified a correlation of mutDFNA5 with processes related to the ER and protein folding. The latter observation was not only present in *Saccharomyces cerevisiae*, but could also be confirmed in human cell lines. Furthermore, we show that the MAPK pathways, namely the induction of the extracellular signal-regulated kinases (*ERK*) and c-Jun N-terminal kinase (*JNK*), are activated upon mut*DFNA5* transfection in human cell lines.

At this specific moment the exact role of the mitochondria and ER remains unclear. These two organelles do not exist as separate entities, but physically interact by mitochondria-associated membranes [10]. Thereby our data suggest the presence of a cellular adaptive response related to mitochondrial bio-energetics which could be associated with the ER. The exact correlation between these two processes and *DFNA5* is still a matter of debate but further elucidation will lead to a better understanding of *DFNA5*-induced cell death mechanisms.

3.3 Material and methods

3.3.1 Yeast

3.3.1.1 Yeast strains and growth conditions

In this study, we used the BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) wild-type strain [11]. Full-length cDNA of either wt*DFNA5* or mut*DFNA5* was isolated and amplified as previously described [8]. Amplified products were ligated into yeast pYX212 plasmid containing a HA-marker (Clontech, Mountain View, CA, USA) using *EcoR*I and *BamH*I restriction sites. All constructs were verified by bidirectional sequencing on an ABI genetic analyser 3130xl (AppliedBiosystems, FosterCity, CA, USA).

Yeast strains were grown at 30°C in selective medium containing 2% glucose (SD-URA). 50 ml yeast cultures, transformed with either wt*DFNA5* or mut*DFNA5* were harvested in mid-exponential phase (OD_{600nm}=3.5-4.2) and at the post-diauxic shift (OD_{600nm}=7.4-8.4). Standard transformation techniques were applied for these transformations [12].

3.3.1.2 RNA extraction yeast

RNA was collected from yeast at mid-exponential phase and just after the post-diauxic shift using RNA pure kit following the manufactures instructions (GenHunter® Corporation, Nashville, TN, USA). All steps were performed in duplo at 4°C to avoid RNA degradation. This resulted eventually in eight different RNA samples: mid-exponential wt*DFNA5*, (WT_E1 and WT_E2), mid-exponential mut*DFNA5* (Mut_E1 and Mut_E2), post-diauxic wt*DFNA5* (WT_S1 and WT_S2) and post-diauxic mut*DFNA5* (Mut_S1 and Mut_S2).

3.3.1.3 Microarray design and analysis

Microarray experiments were performed at the VIB Nucleomics Core (www.nucleomics.be). Before labelling, RNA concentration and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyser 2100 (Agilent). Per sample, an amount of 1 µg of total RNA spiked with 10 viral polyA transcript controls (Agilent, Santa Clara, CA, USA) was converted to double stranded cDNA in a reverse transcription reaction. Subsequently the samples were converted to antisense cRNA, amplified and labelled with Cyanine 3-CTP (Cy3) or Cyanine 5-CTP (Cy5) in an in vitro transcription reaction according to the manufacturer's protocol (Agilent, Santa Clara, CA, USA). A mixture of purified and labelled cRNA (Cy3 label: 5 pmol; Cy5 label: 3.5 pmol) was hybridised on Agilent Yeastv2 arrays followed by washing, according to the manufacturer's procedures. To assess the raw probe signal intensities, arrays were scanned using the Agilent DNA MicroArray Scanner and probe signals were quantified using Agilent's Feature Extractor software (version 10.5.1.1).

In total four different comparisons were made, and each comparison was done in duplo by a colour flip. The different comparisons are shown in Fig. 1. Gene expressions of strains transformed with wt*DFNA5* were compared to

strains transformed with mut*DFNA5*, both in mid-exponential phase (comparison 1a) and at the post-diauxic shift (comparison 1b). Additionally, comparisons were made between the mid-exponential and the post-diauxic shift of either wt*DFNA5*- (comparison 2b) or mut*DFNA5*- (comparison 2a) transformed cells.

Analysis of the microarray data was performed using the R package LIMMA (http://www.bioconductor.org) [13]. Fold changes were computed using raw Cy3 and Cy5 intensities values provided by Agilent's Feature Extractor software (version 10.5.1.1) and loess normalisation and background correction were performed to determine the log2-ratios per array. Differential expression was assessed via the moderated t-statistic, described in Smyth *et al.* [14]. To control the false discovery rate, multiple testing correction was performed. This eventually generated four differentially expressed gene lists.

3.3.2 HEK293T cells

3.3.2.1 Cell culture and growth conditions

The microarray experiments in human cell lines were performed using Human Embryonic Kidney 293T cells (HEK293T). HEK293T cells were subcultured in 60 mm dishes at a density of $2x10^6$ cells in Dulbecco's modified Eagle's medium containing 4500 mg/L glucose supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Overnight cultures were transfected with either wt*DFNA5* or mut*DFNA5* using lipofectamine. Six hours post-transfection cells were harvested using Triple-x reagent for RNA extraction. All products for human cell line cultures were obtained from Invitrogen (San Diego, CA, USA). Full-length cDNA of either wt*DFNA5* or mut*DFNA5* was isolated and amplified as previously described [8].

3.3.2.2 RNA extraction

Qiagens RNeasy mini kit was used for RNA extraction from transfected HEK293T cells (Qiagen, Hilden, Germany) at different time-points. For the microarray experiment RNA was extracted twelve hours post-transfection, for the gene analysis by real-time rtPCR, RNA was extracted at either 3, 6,

12, 15, 18, 20 or 24 hours post-transfection. For the microarray experiment, the integrity of the resulting RNA was checked on an automated Experion electrophoresis system (Biorad, Hercules, CA, USA).

3.3.2.3 Microarray design and analysis

The 'Totalprep RNA Amplification' kit was used to amplify the RNA samples (Illumina, Ambion, Austin, TX, USA). Doublestranded-cDNA was generated from the mRNA fractions followed by an in vitro transcription reaction which produced cRNA strands containing biotin-UTP nucleotides. 750 ng of the resulting cRNA samples were hybridised to an Illumina human HT12v3 beadchip (Illumina, San Diego, CA, USA).

Six independent biological replicates were used for either wt*DFNA5*- or mut*DFNA5*-transfected HEK293T cells and loaded on the chip. Overnight hybridisation at 58°C was followed by washing and a streptavidin-Cy3 dye labelling (Amersham, Buckinghamshire, England). An Illumina Iscan equipped with Iscan control software was used to measure the intensity values and XY coordinates for every probe on the array. The resulting data files were then analysed using the R package "Beadarray v1.14.0" [15] followed by a quality control and quantile normalisation. LIMMAv3.2.1 was used for the further analysis of the normalised intensity values to determine the differentially expressed genes [14].

3.3.2.4 Real-time rtPCR

To confirm the results obtained from the microarray data in HEK293T cells, gene expression was studied in human HEK293T cell lines using Power SYBR Green RNA-to CT 1 Step Kit (Invitrogen, San Diego, CA, USA). Each reaction mixture contained 200 nM final primer concentration (primer pairs are shown in Suppl Data Table 3.1) and 30 ng RNA template. All reactions were performed in triplicate on a LightCycler 480 system (Roche, Basel, Switzerland) and resulting data were analysed by Qbase plus (Biogazelle, Ghent, Belgium). At least three housekeeping genes were used each time as a reference, namely *HMBS*, *GAPDH*, *RPL13A*, *UBC* and *YWHAZ* (Suppl. Data Table 3.1) [16].

3.3.2.5 Western blot analysis

For western blotting, cells were lysed using RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Pierce, Rockford, IL, USA) containing a PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche), an EDTA-free protease tablet and 10 µl (25 units/µl) benzonase (70746-4 Novagen®, Merck Millipore, Darmstadt, Germany). Transfected HEK293T cells were lysed for 20 minutes at 4°C and centrifuged at 2000 *g* at 4°C to obtain protein lysates.

Proteins were electrophoretically separated and blotted onto a nitrocellulose membrane (Whatman, Kent, UK). This membrane was blocked for one hour in 5% non-fat dry milk and afterwards incubated overnight (4°C) in one of the following primary antibodies: anti-phospho-SAPK/JNK (Thr183/Tyr185, #4668), anti-phospho-p44/42 (ERK1/2, #4370), anti-SAPK/JNK (#9252), anti-p44/42 (ERK1/2, #9102) (Cell Signalling Technologies, MA, USA) or anti-β-Actin (A5316, Sigma Aldrich, MO, USA). After washing, the membranes were incubated with either a secondary goat anti-rabbit (ab6721, Abcam, Cambridge, UK) or sheep anti-mouse (NA931, GE Healthcare, Buckingmersham, UK) antibody. Finally, the corresponding proteins were visualised using Enhanced ChemiLuminescence Western Blotting Substrate (Thermo Scientific, IL, USA). Quantification of the protein levels was performed using the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK).

3.3.3 Gene ontology analysis

In addition to the determination of the differentially expressed genes, gene ontology (GO) analysis was performed in order to identify enriched GO annotations. GO analysis determines the biologically, cellular and molecularly enriched GO terms linked to the differentially expressed genes. We used an open-source application, the Ontologizer (http://compbio.charite.de/contao/index.php/ontologizer2.html), as a tool to statistically analyse the high-throughput data [17]. A standard method for statistics, the “Term-for-Term analysis”, was used followed by Benjamini-Hochberg correction for multiple testing [18, 19]. Differentially expressed

genes with a corrected p-value below 0.05 and a log2 (fold change) of 1.5 and 0.5, in respectively yeast and human cell lines, were selected for this analysis.

3.4 Results

To study the biological differences of cell death induced by *DFNA5* in *Saccharomyces cerevisiae*, we performed a transcriptomic study. RNA samples were collected in duplo in the mid-exponential phase and at the post-diauxic shift of yeast cells transformed with either wt*DFNA5* of mut*DFNA5*. Figure 1 shows all the comparisons studied between the different RNA samples (comparisons are labelled 1a, 1b, 2a and 2b). Analysis of the microarray data generated four lists of differentially expressed genes. Investigation of the differentially expressed genes in mid-exponential phase (comparison 1a) revealed no significantly up- or down-regulated genes. Therefore this comparison was excluded and only comparisons 1b, 2a and 2b (shown in Fig. 1) will be taken into account and described.

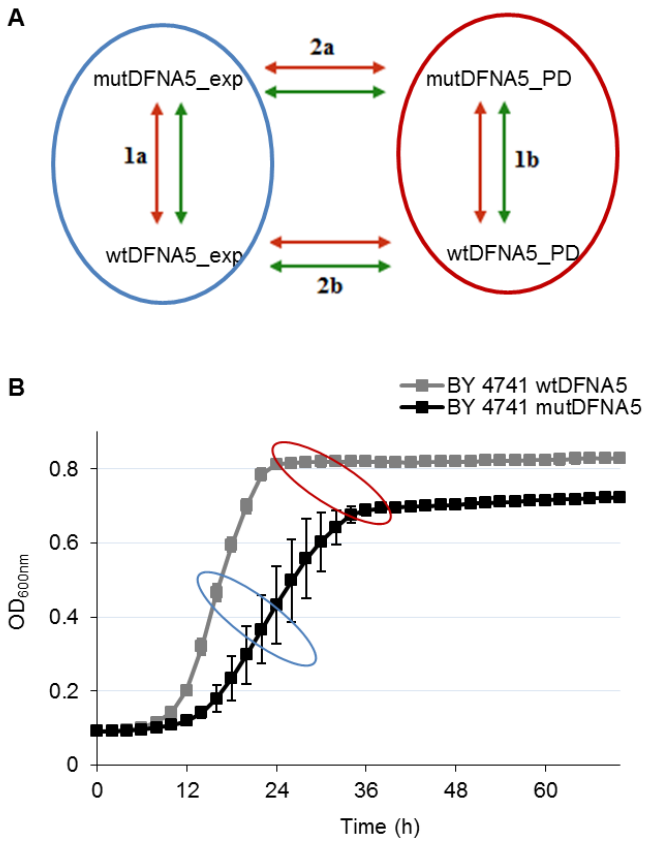

Fig. 1: Yeast microarray design. **a.** Illustration of the different comparisons that were studied between the RNA samples. RNA was collected from yeast strains transformed with either wt*DFNA5* or mut*DFNA5* at two different time-points. The red and green lines represent the colour flip. **b**. Growth profile of the *Saccharomyces cerevisiae* BY4741 background strain transformed with either wt*DFNA5* (grey squares) or mut*DFNA5* (black squares). The blue and red ellipse illustrate respectively the mid-exponential and post-diauxic time-points when RNA was collected.

3.4.1 Mitochondria-related processes are up-regulated in mut*DFNA5* in post-diauxic shift

Comparison of the differentially expressed genes in the post-diauxic shift (1b) resulted in 451 significantly up-regulated genes between yeast expressing mut*DFNA5* and using yeast expressing wt*DFNA5* as a reference. The top 34 of the highest differentially up-regulated genes at the post-diauxic shift and their association with a specific GO term is shown in Table 1. Differentially expressed genes with a log2 (fold change) equal or higher than 1.5 were selected for GO-enriched annotations, generating 85 significantly up-regulated genes, which resulted in 65 significantly up-regulated enriched GO terms.

Analysis of the biological, cellular and molecular GO annotations confirmed the role of the mitochondria in mut*DFNA5*-induced cell death (Suppl. Data Table 3.2, indicated in red). Analysis of the GO annotations revealed that the molecular cytochrome c oxidase activity-related process (GO: 0004129) was the most significantly mitochondrial associated process. Further down the list, several biological, molecular and cellular GO processes related to mitochondrial mechanisms, such as mitochondrial ATP synthesis-coupled electron transport (GO:0042775), aerobic respiration (GO:0009060), the mitochondrial respiratory chain (GO:0005746), mitochondrial respiratory chain complex IV (GO:0005751) and oxidative phosphorylation (GO:0006119) were significantly associated suggesting mitochondrial dysfunction (Suppl. Data Table 3.2).

Table 1: Top 34 of the highest differentially up-regulated genes upon transfection of mut*DFNA5* in yeast at the post-diauxic shift

Gene name	logFC	AveExpr	adj.P.Val	GO Term
MAL12	4.16	9.08	0.04	maltose metabolic process
COX3	4.13	7.46	0.00	respiratory electron transport chain
MAL32	3.91	8.19	0.04	maltose metabolic process
ANB1	3.60	9.59	0.01	positive regulation of translational elongation
SLZ1	3.08	6.38	0.00	-
PRM7	3.07	11.62	0.01	-
YPR077C	3.05	9.44	0.00	-
PRM7	3.04	11.01	0.01	-
HXT7	3.02	14.80	0.03	transmembrane transport
YDR374C	2.94	6.69	0.02	-
YPL014W	2.89	8.59	0.02	protein phosphorylation
AI5_ALPHA	2.89	10.49	0.04	movement of group I intron (COXI gene)
RTA1	2.83	7.90	0.00	response to stress
YPR078C	2.65	7.06	0.00	-
YLR194C	2.45	13.79	0.01	fungal-type cell wall organization
SUC2	2.44	11.79	0.04	carbohydrate metabolic process
SPO20	2.43	9.06	0.01	protein binding
YOL047C	2.39	10.40	0.01	integral component of membrane
Q0297	2.38	7.24	0.01	-
MND1	2.27	7.03	0.01	reciprocal meiotic recombination
VAR1	2.23	4.18	0.02	mitochondrial translation

Table 1 continued: Top 34 of the highest differentially up-regulated genes upon transfection of mut*DFNA5* in yeast at the post-diauxic shift

Gene name	logFC	AveExpr	adj.P.Val	GO Term
YPL141C	2.22	9.49	0.01	protein phosphorylation
HXT4	2.22	13.42	0.05	substrate-specific transmembrane transporter activity
YNR034W-A	2.20	13.88	0.02	-
YDR034W-B	2.17	9.17	0.02	cell cortex
COX2	2.16	6.19	0.01	mitochondrial electron transport, cytochrome c to oxygen
OLI1	2.15	15.96	0.00	ATP synthesis-coupled proton transport
YBL065W	2.15	6.87	0.01	-
SPR28	2.13	7.20	0.00	cell cycle
COX1	2.12	7.77	0.01	mitochondrial electron transport, cytochrome c to oxygen
DRE2	2.05	12.58	0.01	iron-sulfur cluster assembly
YKR075C	2.01	13.64	0.00	fungal-type cell wall organization
YJL218W	2.00	6.92	0.04	-
YLR338W	1.95	7.52	0.01	-
YGR287C	1.92	8.55	0.01	carbohydrate metabolic process

LogFC, logarithm of fold change, up-regulation in mut*DFNA5* yeast samples using wt*DFNA5* as a reference; adj.p.value, p-value adjusted for multiple hypothesis testing. Genes involved in processes related to the COX activity or energy processes are indicated in bold. '-' denotes a gene with an unknown function. 'Go Term' denotes the biological process annotated to the gene or the molecular process/cellular component if the biological process was unknown. G.O., gene ontology.

Next we compared the identified GO terms with the list containing the highest differentially up-regulated genes generated by the R package LIMMA to evaluate the resemblances (Table 1). As shown in Table 1, several mitochondrial genes related to these GO processes were indeed present in the list, including *COX/1/2/3* and *AI5_ ALPHA* (Table 1, indicated in bold). COX1/2/3 are the three main subunits of the cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport chain, encoded by the mitochondrial genome. The electron transport chain is part of the mitochondrial oxidative phosphorylation pathway providing most of the cellular ATP [20]. AI5_ALPHA is an endonuclease encoding a mobile intron of the *COXI* gene [21, 22]. Up-regulation of the main *COX* genes suggests enhanced COX activity, which has been associated with increased oxidative stress [20, 23].

In addition, two other groups of significantly enriched GO annotations could be distinguished, namely GO annotations related to catabolic and metabolic energy processes, such as oligosaccharide catabolic process (GO:0009313) or maltose catabolic processes (GO:0000025), and mechanisms related to transporter activity, such as cation (GO:0008324) and several sugar (GO:0005353 for example) transmembrane activities (Suppl. Data Table 3.2, indicated in respectively green and blue). Consistent with the previous results, comparison of these processes with the highest differentially up-regulated genes in Table 1 confirmed these identified GO terms (Table 1). Different maltose and sucrose genes like *MAL12*, *MAL32* and *SUC2*, and transmembrane transporter genes like *HXT4/7* were present in the list (Table 1).

These results revealed an important role for mitochondria-related processes in mut*DFNA5* transformed yeast cells in the post-diauxic shift. Especially processes associated with the mitochondrial respiratory chain complex and cytochrome c oxidase activity showed significant differences.

3.4.2 Processes associated with glycolysis are down-regulated in mut*DFNA5* at post-diauxic shift

To investigate the significantly down-regulated processes and genes in the post-diauxic shift between wtDFNA5 and mutDFNA5, we used the same method as in the previous part. This revealed 585 significantly down-

regulated genes in cells expressing mut*DFNA5* as compared to those expressing wt*DFNA5*. The top 34 of the highest differentially down-regulated genes and their association with a specific GO term is shown in Table 2.

Table 2: Top 34 of the highest differentially down-regulated genes upon transfection of mut*DFNA5* in yeast at the post-diauxic shift.

Gene name	logFC	AveExpr	adj.P.Val	GO Term
ENO2	-3.00	16.12	0.01	glycolytic process
SSA2	-2.68	14.66	0.01	-
VHT1	-2.54	13.48	0.02	biotin transport
DET1	-2.44	16.33	0.01	intracellular sterol transport
RPS22A	-2.44	14.70	<0.00	translation
PGK1	-2.39	16.90	0.01	glycolytic process
TDH3	-2.30	15.32	0.01	glucose metabolic process
TPI1	-2.06	17.08	0.01	metabolic process
PDC1	-2.05	16.95	0.03	thiamine pyrophosphate binding
TDH2	-2.02	14.26	0.01	glucose metabolic process
YHR140W	-1.95	8.34	0.03	-
TOS4	-1.94	10.23	0.05	protein binding
YRO2	-1.91	14.89	0.03	ion channel activity
FBA1	-1.91	17.14	0.01	glycolytic process
RPL9A	-1.90	14.27	0.03	translation
RPL22A	-1.86	14.33	0.01	translation
URA7	-1.83	12.90	<0.00	pyrimidine nucleobase biosynthetic process
ERG25	-1.77	14.05	0.05	sterol biosynthetic process
YNR021W	-1.74	11.30	<0.00	-
YBT1	-1.73	12.67	0.05	bile acid and bile salt transport
KAP122	-1.71	9.48	<0.00	protein import into nucleus

Table 2 continued: Top 34 of the highest differentially down-regulated genes upon transfection of mut*DFNA5* in yeast at the post-diauxic shift.

Gene name	logFC	AveExpr	adj.P.Val	GO Term
CDC19	-1.71	16.94	0.02	pyruvate metabolic process
YDR509W	-1.70	6.06	<0.00	-
RPS9B	-1.67	15.08	<0.00	translation
GNP1	-1.64	12.19	0.01	amino acid transmembrane transport
FTR1	-1.61	13.04	0.04	high-affinity iron ion transport
GPD1	-1.61	13.24	0.05	glycerol-3-phosphate dehydrogenase [NAD+] activity
RPL16B	-1.61	15.38	0.01	translation
YGR266W	-1.61	9.17	0.03	-
RPA43	-1.60	10.54	<0.00	transcription, DNA-templated
ARC1	-1.59	13.85	0.01	tRNA aminoacylation for protein translation
PGI1	-1.58	16.09	0.03	glycolytic process
RPL18B	-1.57	9.61	0.01	translation
SPE3	-1.56	12.70	0.01	catalytic activity

LogFC, logarithm of fold change, down-regulation in mut*DFNA5* yeast samples using wt*DFNA5* as a reference; adj.p.value, p-value adjusted for multiple hypothesis testing. '-' denotes a gene with an unknown function. 'Go Term' denotes the biological process annotated to the gene or the molecular process/cellular component if the biological process was unknown. Genes involved in processes related to the glycolysis or the pentose phosphate pathway are indicated in bold. G.O., gene ontology.

The significantly down-regulated biological GO annotations can be divided in two main groups (Suppl. Data Table 3.3). One group is related to ribosomal processes and hence translation such as cytosolic ribosome (GO:0022626) and the positive regulation of translation fidelity (GO:0045903) (Suppl. Data Table 3.3, indicated in red). The second group was correlated with the biosynthesis and metabolism of glucose (GO:0006007), monosaccharide (GO:0046365) and glycolysis (GO:0006096).

Again, we compared the identified GO terms with the list containing the highest differentially down-regulated genes generated by the R package LIMMA to evaluate the resemblances (Table 2). As expected, this list contained several components associated with the glycolysis and several protein components of the small and large ribosomal subunit.

Interestingly, the list also contained several genes such as, *TPI*, *TDH2/3, PGK1* and *CDC19*, which are all enzymes playing a role in either the glycolytic or the pentose phosphate pathway (PPP) (Table 2, indicated in bold). *CDC19* is the yeast homologue of the human pyruvate kinase (PK) gene. Down-regulation of PK has been correlated with the activation of the PPP and the redirection of the metabolic flux from glycolysis to PPP both in human cell lines and in yeast [24-26]. This will enhance the anti-oxidant response and hence increases the tolerance for oxidative stress [26-28]. The down-regulation of genes involved in glycolysis and the PPP could suggest a link with oxidative stress providing a protection mechanism for mut*DFNA5*-transformed yeast cells

3.4.3 Induction of ER-related processes upon mutDFNA5 expression in yeast

In addition to the comparison of mut*DFNA5* and wt*DFNA5* in the post-diauxic shift, the modifications between mid-exponential phase and at the post-diauxic shift were also investigated both in mut*DFNA5*- (comparison 2a Fig. 1) and wt*DFNA5*- (comparison 2b Fig. 1) transformed yeast cells. As both comparisons studied the differentially expressed genes in exponential phase versus post-diauxic shift, we expect the presence of many similar significantly expressed genes. These genes are probably related to the post-diauxc shift but not due to mut*DFNA5* expression. Therefore, genes which were differentially expressed at the post-diauxic shift upon mut*DFNA5*

transformation, but do not show any differences upon wt*DFNA5* expression in post-diauxic phase, are potentially related to mut*DFNA5*-associated processes. These were assigned as mut*DFNA5*-related changes not associated with the post-diauxic shift in yeast.

The GO-enriched processes significantly associated with up-regulated genes were very similar between wt*DFNA5*- and in mut*DFNA5*-transformed yeast cells and correlated with translation. GO-enriched terms significantly associated with down-regulated genes were related to ribosomes and RNA. These processes were probably due to the shift to respiration and not in particular related to mut*DFNA5* (data not shown). However, three main classes could be distinguished at the post-diauxic shift. Two of them were more prominent in mut*DFNA5*-transformed yeast cells. One class was related to the biosynthesis and the metabolism of lipids (GO:0008610), such as (ergo)sterols (GO:0016126), (phyto)steroids (GO:0006694) and fatty acids (GO:0006633) (Suppl. Data Table 3.4, indicated in red). The other group was associated with endoplasmic reticulum processes (GO:0005783), such as the endoplasmic reticulum membrane (GO:0005789) and the Sec62/63 complex (GO:0031207) (Suppl. Data Table 3.4, indicated in green).

The third group which could be distinguished was related to the cytoskeleton (GO:0005856) and more pronounced in wt*DFNA5* transformed yeast cells. Cellular enriched GO terms such as the microtubule cytoskeleton (GO:0015630) and the microtubule organizing centre (GO:0005815) were present in this list (Suppl. Data Table 3.5, indicated in red).

3.4.4 Association of DFNA5 with the MAPK-related mechanisms in HEK293T cells

To evaluate the value of the yeast model and to further elucidate the *DFNA5*-related pathways, a microarray experiment was performed in human HEK293T cells. As described previously, mut*DFNA5* induced a growth defect in transfected HEK293T cells compared to wt*DFNA5* and control [7]. These cell death events were evident from 9 h post-transfection and peaked at 12 h (data not shown). Therefore RNA samples of HEK293T cells were collected 12 h post-transfection. A transcriptomic analysis was performed on HEK293T cells transfected with either wt*DFNA5* or mut*DFNA5*. Six biological replicates of every RNA sample were collected although one wt*DFNA5*-transfected

sample did not survive quality control. Therefore, subsequent analyses, using wt*DFNA5* as a reference, were performed on five wt*DFNA5*- versus six mut*DFNA5*-transfected samples. Analysis using "Beadarray" and "LIMMA" packages available in R identified 228 significantly up- and 222 significantly down-regulated genes after correction for multiple hypothesis testing ($p<0.05$). Besides individual gene expression, GO analysis was performed to determine the biologically, cellular and molecularly enriched GO annotations linked to the differentially expressed genes.

Table 3 shows the top 34 of the highest significantly up-regulated genes and their association with a specific GO term. It contains several genes related to the MAPK pathway such as *EGR1/*2, *FOSB* and *JUNB* (indicated in bold). Interestingly, this list also contained the *PMAIP1* gene. *PMAIP1* encodes a BH3-only protein belonging to the BCL2 protein family, a family of important regulators of apoptotic cell death related to the mitochondria. The top 34 of the highest down-regulated genes and their association with a specific GO term is shown in Table 4 and contains several genes related to protein folding such as *HSPA6*, *ATF3* and *CTH* (indicated in bold, Table 4).

Table 3: Top 34 of the highest significantly up-regulated genes in mut*DFNA5*-transfected HEK293T cell

Gene name	logFC	AveExpr	adj.P.Val	GO term
ZCCHC12	2.63	12.07	<0.00	transcription, DNA-templated
ETV5	1.88	9.11	<0.00	regulation of transcription, DNA-templated
LOC387763	1.86	10.00	<0.00	-
EGR1	1.83	11.82	<0.00	cellular response to growth factor stimulus, response to cAMP
ARC	1.61	8.76	<0.00	cytoskeleton organization
FOSB	1.58	8.85	<0.00	response to cAMP
KCTD12	1.40	10.32	<0.00	protein homo-oligomerisation
JUNB	1.40	9.09	<0.00	response to cAMP
ADAMTS1	1.24	9.88	<0.00	proteolysis
EGR2	1.19	8.12	<0.00	cellular response to growth factor stimulus, response to cAMP
FOS	1.18	10.17	<0.00	stress-activated MAPK cascade
CITED1	1.16	11.66	<0.00	melanocyte differentiation
MAFB	1.12	9.32	<0.00	negative regulation of erythrocyte differentiation
RPL15	1.11	13.23	<0.08	translation
GPR3	1.07	8.29	<0.00	G-protein-coupled receptor signalling pathway
TNFRSF12A	1.06	9.77	<0.00	positive regulation of apoptotic process
TRIB1	1.04	11.60	<0.00	regulation of MAP kinase activity
ANXA1	1.03	8.98	<0.00	inflammatory response
PMAIP1(2750367)	1.00	10.76	<0.00	positive regulation of apoptotic process
ETV4	0.99	7.71	<0.00	regulation of transcription, DNA-templated
SPRY2	0.97	9.96	<0.00	positive regulation of protein serine/threonine kinase activity
PMAIP1(6020598)	0.96	9.30	<0.00	positive regulation of apoptotic process
CCNA1	0.94	7.60	<0.00	regulation of cell cycle

Table 3 continued: Top 34 of the highest significantly up-regulated genes in mut*DFNA5*-transfected HEK293T cell

Gene name	logFC	AveExpr	adj.P.Val	GO term
TRIM9	0.92	10.00	<0.00	protein ubiquitination
MAFF	0.90	7.95	<0.00	regulation of transcription, DNA-templated
FAM84B	0.90	12.04	<0.00	protein binding
EPHA2	0.89	7.98	<0.00	multicellular organismal development
CYP1B1	0.88	8.52	<0.00	membrane lipid catabolic process
NDRG1	0.87	9.54	<0.00	DNA damage response, signal transduction by p53 class mediator
SERTAD1	0.85	10.14	<0.00	transcription, DNA-templated
MCL1	0.81	10.20	<0.00	apoptotic mitochondrial changes
SPRY4	0.81	7.66	<0.00	multicellular organismal development
VGF	0.79	8.30	<0.00	growth factor activity
NEFM	0.79	11.58	<0.00	neurofilament cytoskeleton organization

LogFC, logarithm of fold change, down-regulation in mut*DFNA5*-transfected HEK293T cells using wt*DFNA5* as a reference; adj.p.value, p-value adjusted for multiple hypothesis testing. 'Go Term' denotes the biological process annotated to the gene or the molecular process/cellular component if the biological process was unknown. '-' denotes a gene with an unknown function. '(number)' denotes the array address of the specific splice variant on the microarray chip. Genes involved in processes related to either the MAPK pathway, cAMP response or the mitochondria are indicated in bold. G.O., gene ontology.

Table 4: Top 34 of the highest significantly down-regulated genes in mut*DFNA5*-transfected HEK293T cells

Gene name	logFC	AveExpr	adj.P.Val	GO term
HSPA6 (160092)	-1.54	10.49	<0.00	response to unfolded protein
DDIT4	-1.26	10.18	<0.00	intrinsic apoptotic signalling pathway in response to DNA damage by p53 class mediator
CLEC2D	-0.97	9.47	<0.00	cell surface receptor signalling pathway
ZNF256	-0.91	9.10	<0.00	transcription, DNA-templated
TDP1	-0.87	10.90	<0.00	DNA repair
LRP5L	-0.84	8.76	<0.00	canonical Wnt signalling pathway
CTH (60138)	-0.80	10.40	<0.00	endoplasmic reticulum-unfolded protein response
SNORD15B	-0.78	8.76	<0.00	-
C17orf48	-0.76	8.45	<0.00	hydrolase activity
LOC389816	-0.73	8.57	<0.00	ion transport
HSPA6 (1710553)	-0.71	9.28	<0.00	Response to unfolded protein
ZMAT3	-0.70	12.59	<0.00	apoptotic process
PMS2L3	-0.67	8.69	<0.00	-
PCDHGA9	-0.66	7.61	<0.00	homophilic cell adhesion
CTH (6220504)	-0.65	8.29	<0.00	endoplasmic reticulum-unfolded protein response
C3orf34	-0.64	12.64	<0.00	centriole
FANCE	-0.64	9.35	<0.00	DNA repair
HRK	-0.64	8.82	<0.00	positive regulation of release of cytochrome c from mitochondria
C5orf13	-0.64	9.27	<0.00	regulation of neuron differentiation
LRAP	-0.63	13.23	<0.00	proteolysis
CLEC2D	-0.61	14.72	<0.00	cell surface receptor signalling pathway
GUSBL1	-0.60	10.04	<0.00	-
DTL	-0.60	10.17	<0.00	protein polyubiquitination

Table 4 continued: Top 34 of the highest significantly down-regulated genes in mut*DFNA5* transfected HEK293T cells

Gene name	logFC	AveExpr	adj.P.Val	GO term
ZSCAN16	-0.58	8.66	<0.00	transcription, DNA-templated
CTH (1470576)	-0.58	8.75	<0.00	endoplasmic reticulum-unfolded protein response
LOC654194	-0.58	13.17	<0.00	-
SNORD68	-0.58	10.17	<0.00	-
ZNF205	-0.57	7.83	<0.00	transcription, DNA-templated
ZNF416	-0.57	8.56	<0.00	transcription, DNA-templated
LRRC26	-0.57	8.19	<0.00	ion transport
Hs.537645	-0.57	7.54	<0.00	-
ATF3 (4780128)	-0.56	11.95	<0.00	activation of signalling protein activity involved in unfolded protein response
ACOT2	-0.55	9.15	<0.00	long-chain fatty acid metabolic process
PCK2	-0.55	9.22	<0.00	gluconeogenesis
LRRC20	-0.54	8.67	<0.00	

LogFC, logarithm of fold change, down-regulation in mut*DFNA5*-transfected HEK293T cells using wt*DFNA5* as a reference; adj.p.value, p-value adjusted for multiple hypothesis testing. 'Go Term' denotes the biological process annotated to the gene or the molecular process/cellular component if the biological process was unknown. '-' denotes a gene with an unknown function. '(number)' denotes the array address of the specific splice variant on the microarray chip. Genes involved in processes related to protein folding are indicated in bold. G.O., gene ontology.

Subsequent GO analysis of the biological annotations revealed, in addition to the more general development processes, the up-regulation of the MAPK pathway (GO:0043407) and the cAMP response (GO:0051591) (Suppl. Data Table 3.6, indicated in red). The response to protein folding (GO:0006986) and to topologically incorrect protein (GO:0035966) were the only two processes associated with the down-regulated genes in human cell lines and both were related to protein folding (Suppl. Data Table 3.7). The most important genes that are involved in these processes were *HSPA6*, a heat shock protein and several chaperones proteins, such as *DNAJB1* and *DNAJB2*.

These results demonstrate the association of mut*DFNA5*-induced cell death with the MAPK pathways. The identification of processes related to protein folding supports the results in yeast where GO terms related to the ER were significantly associated with mutDFNA5.

3.4.5 Validation of the MAPK role in DFNA5-related cell death in HEK293T cells

The data generated by the transcriptomic analysis in HEK293T cells were validated by real-time rtPCR on newly collected RNA samples. *EGR1* and *FOSB* gene expression was investigated on different time-points ranging from 3 h until 18 h post-transfection. As shown in Fig. 2, significantly up-regulated *EGR1* and *FOSB* gene expression was observed from 12 h until 18 h post-transfection ($p<0.05$) (Fig. 2). Hence the data generated by the transcriptomic analyses were indeed confirmed by real-time rtPCR as demonstrated by up-regulation of genes related to the MAPK pathway.

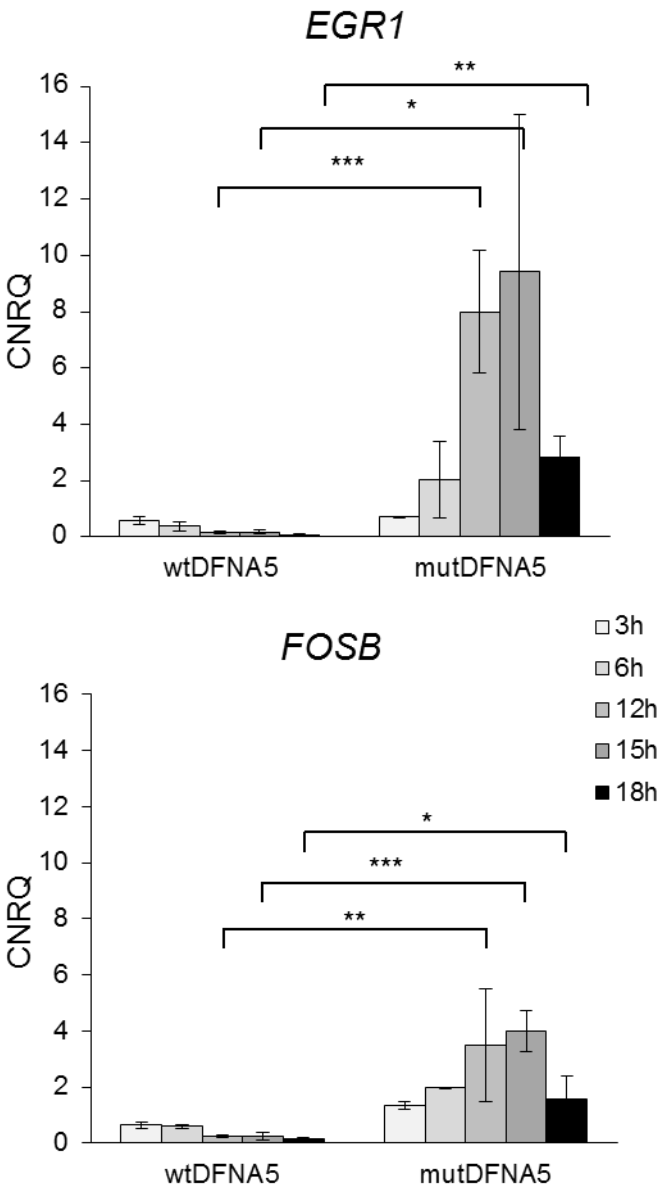

Fig. 2: Increased *EGR1* and *FOSB* gene expression in mut*DFNA5*-transfected HEK293T cells. RNA samples were collected from HEK293T cells transfected with either wt*DFNA5* or mut*DFNA5* and gene expression was measured by real-time rtPCR. Significantly increased expression was seen in mut*DFNA5* at 12 h (p(egr1)=0.000; p(fosB)=0.006), 15 h (p(egr1)=0.017; p(fosB)=0.000) and 18 h (p(egr1)=0.004; p(fosB)=0.026) post-transfection. *: p<0.05; **: p<0.01; ***: p<0.001.

After confirmation by real-time rtPCR, the significance of the activated MAPK pathway was further validated by two independent experiments. It was previously shown that mutDFNA5 induced a growth defect in HEK293T cells compared to wtDFNA5 and control [7]. To investigate the significance of the MAPK pathway, we wondered whether inhibition of the MAPK pathway would attenuate this mutDFNA5-induced growth defect.

Therefore, a specific JNK inhibitor, namely SP600125, was added followed by a viability assay to determine the effect on cell survival. Different concentrations of the JNK inhibitor SP600125 were used to measure viability by flow cytometry and these results were compared to untreated mut*DFNA5*-transfected HEK293T cells. Overnight treatment of the cells with different concentrations of SP600125 did not have any major effect on transfection efficiency, but significantly increased the viability of mut*DFNA5*-transfected cells. Although addition of both 12.5 µM and 25 µM SP600125 both significantly increased the viability with a p-value of respectively 0.020 and 0.004, SP600125 had the greatest effect with a concentration of 25 µM SP600125 where the viability was raised from 31.93% to 51.00% (Fig. 3).

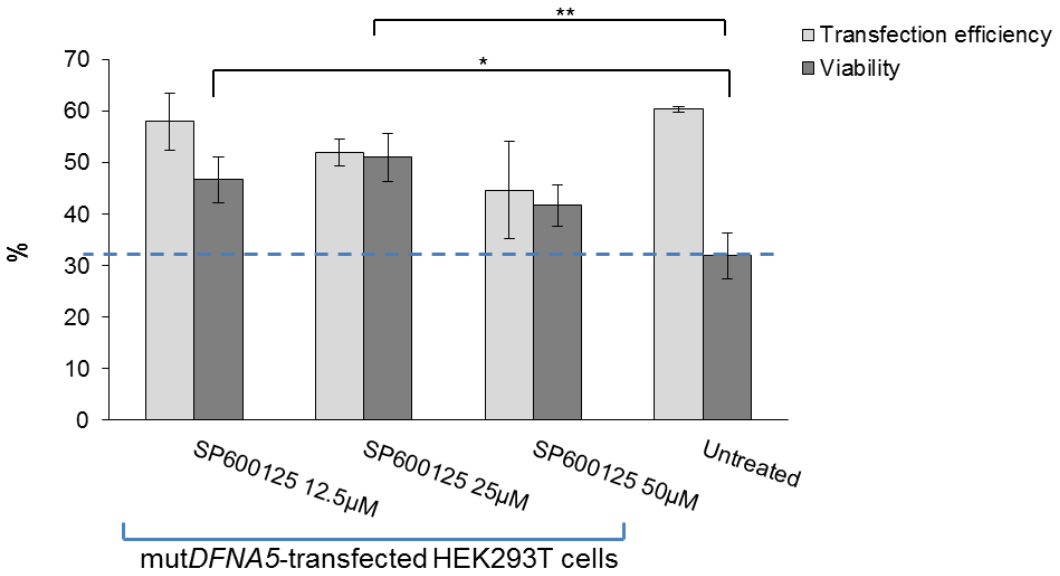

Fig. 3: MAPK inhibitor effect on mut*DFNA5*-transfected HEK293T cells. Mut*DFNA5*-transfected HEK293T cells were pretreated with different amounts of SP600125 (JNK inhibitor). Cell viability was measured and compared to untreated mut*DFNA5*-transfected HEK293T cells. *: $p<0.05$; **: $p<0.01$.

Next, to evaluate the effect of MAPK up-regulation on protein level, different MAPK proteins were studied by western blotting. There are three main MAPK pathways in human cell lines represented by the ERK, JNK and p38 MAPK branch. Consistent with the results obtained by RT-PCR and the viability assay, activation of the MAPK pathway proteins was also demonstrated by western blotting. Total protein lysates were collected from HEK293T cells 12 h post-transfection. Three phosphorylated (activated) and non-phosphorylated (not activated) proteins of the MAPK pathway were studied using six different antibodies. Activation of ERK (p42/p44) and JNK was seen upon mut*DFNA5* transfection compared to control and wt*DFNA5* (Fig. 4a). No differences were seen in the expression level of non-phosphorylated ERK and JNK (Fig. 4a). The expression of p38 was also evaluated but no difference in protein expression was observed between mutDFNA5 compared to wtDFNA5 and control (data not shown). β-Actin was used as a loading control and quantification of the protein expression was performed in relation to β-actin using ImageQuantTL software (GE Healthcare), although this increase was not significant as determined by one way Anova ($p<0.05$, Fig. 4b).

These results suggest that DFNA5 induces cell death mediated through activation of the MAPK pathways. Addition of a MAPK inhibitor partially attenuated the mutDFNA5-induced growth defect identifying the MAPK pathway as an early event in mutDFNA5-associated cell death.

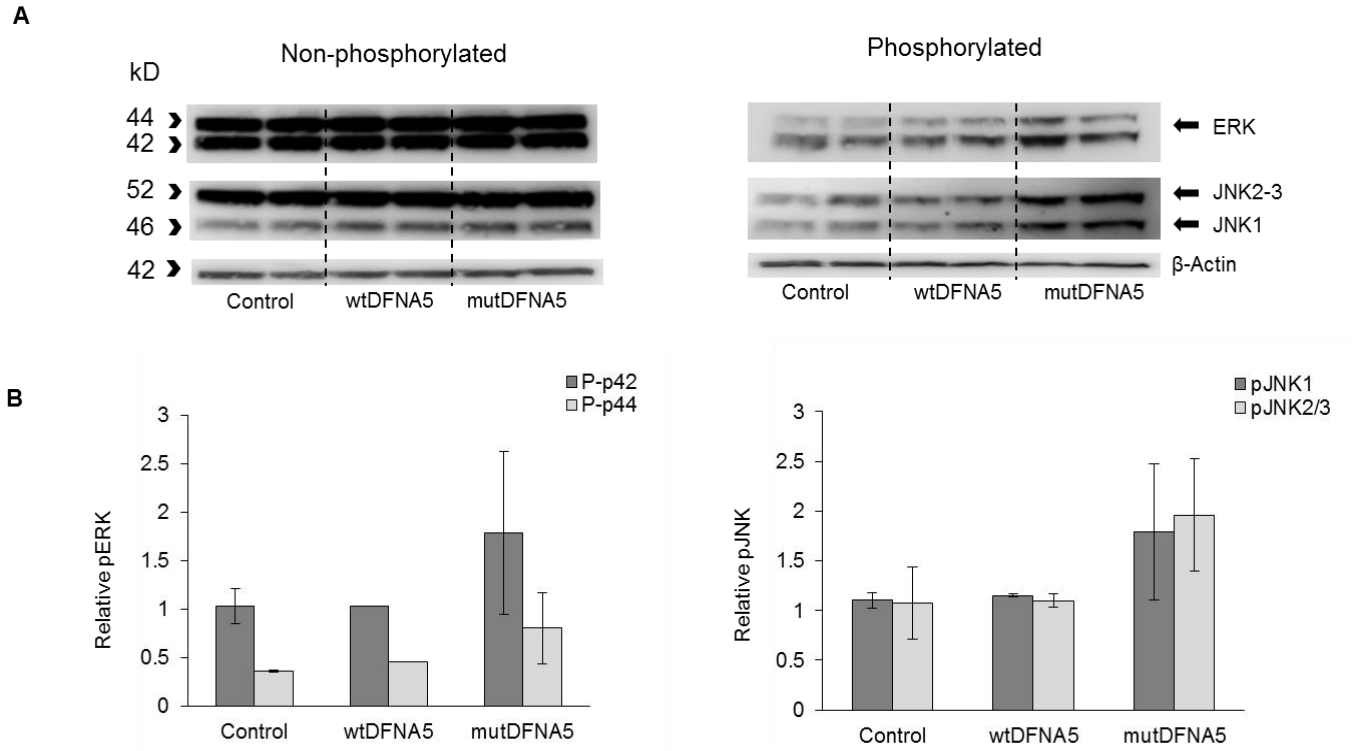

Fig. 4: Activation of the MAPK pathway by mutDFNA5. a. Western blot analysis of phosphorylated and non-phosphorylated ERK (p42/p42) and JNK showing increased activation compared to control and wtDFNA5. **b.** Quantification of the phosphorylated JNK and ERK expression in relation to β-actin.

3.4.6 Comparison of the yeast microarray results with the gene expression in human cell lines

In order to study the significance of the yeast results, a comparison was made between the two microarray experiments. Human homologues of the significantly up- and down-regulated yeast genes at the post-diauxic shift (comparison 1b) were identified using Ensemble Biomart. Of the 451 significantly up- and 585 significantly down-regulated yeast genes, respectively 296 and 647 human homologues were identified. These specific human homologues were analysed by the R package LIMMA generating a new list of human genes. The FC cut-off of the resulting gene list was set to FC 1.2, resulting in 16 up- and 14 down-regulated human genes. *TM7SF2*, *UCP2* and *VPS33B*, three down-regulated human genes, were selected to verify the yeast results in human cell lines using RT-PCR. *UCP2* and *VPS33B* were the two highest down-regulated genes present in the list and therefore selected. UCP2, an uncoupling protein, is a mitochondrial carrier located at the mitochondrial inner membrane. Suppression of UCP2 has been linked to increased ROS production [29, 30] and lifespan regulation [31, 32]. The Vacuolar protein sorting 33 homologue (VPS33B) gene is involved in intracellular vesicle Golgi-to-lysosome transport [33, 34]. *TM7SF2* was selected based on its function which showed some resemblances with the yeast results. TM7SF2 is a transmembrane protein present in the endoplasmic reticulum and associated with biosynthesis of cholesterol. In addition to its role in cholesterol synthesis, TM7SF2 appears to be involved in the inflammatory response upon cellular stress [35-38].

To verify the yeast results in human cell lines, RNA was collected from HEK293T cells transfected with either wt*DFNA5* or mut*DFNA5* at different time-points starting at 12 h after transfection as this was the time-point of the human microarray experiment. The *TM7SF2*, *UCP2* and *VPS33B* genes had a fold change of respectively 1.23, 1.31 and 1.27 on the microarray. RT-PCR on RNA samples 12 h post-transfection confirmed these microarray results as all three genes were down-regulated in mut*DFNA5* compared to wt*DFNA5* (Fig. 5a) with fold changes comparable to the microarray (respectively 1.35, 1.48 and 1.59). Although not significantly, these three genes were down-regulated in HEK293T cells 12 h after transfection (Fig.

5a). The down-regulation was still present at 20h, peaked at 24h after transfection and was even significant for TM7SF2 (p:0.01) and UCP2 (p:0.07) at respectively 20 h and 24 h post-transfection (Fig. 5b). Due to this down-regulation, we can conclude that there are some similarities between the yeast and the HEK293T microarray. Discrepancies are seen when looking at the individual genes, but upon study of the different pathways a role for processes related to protein folding were seen in both model systems.

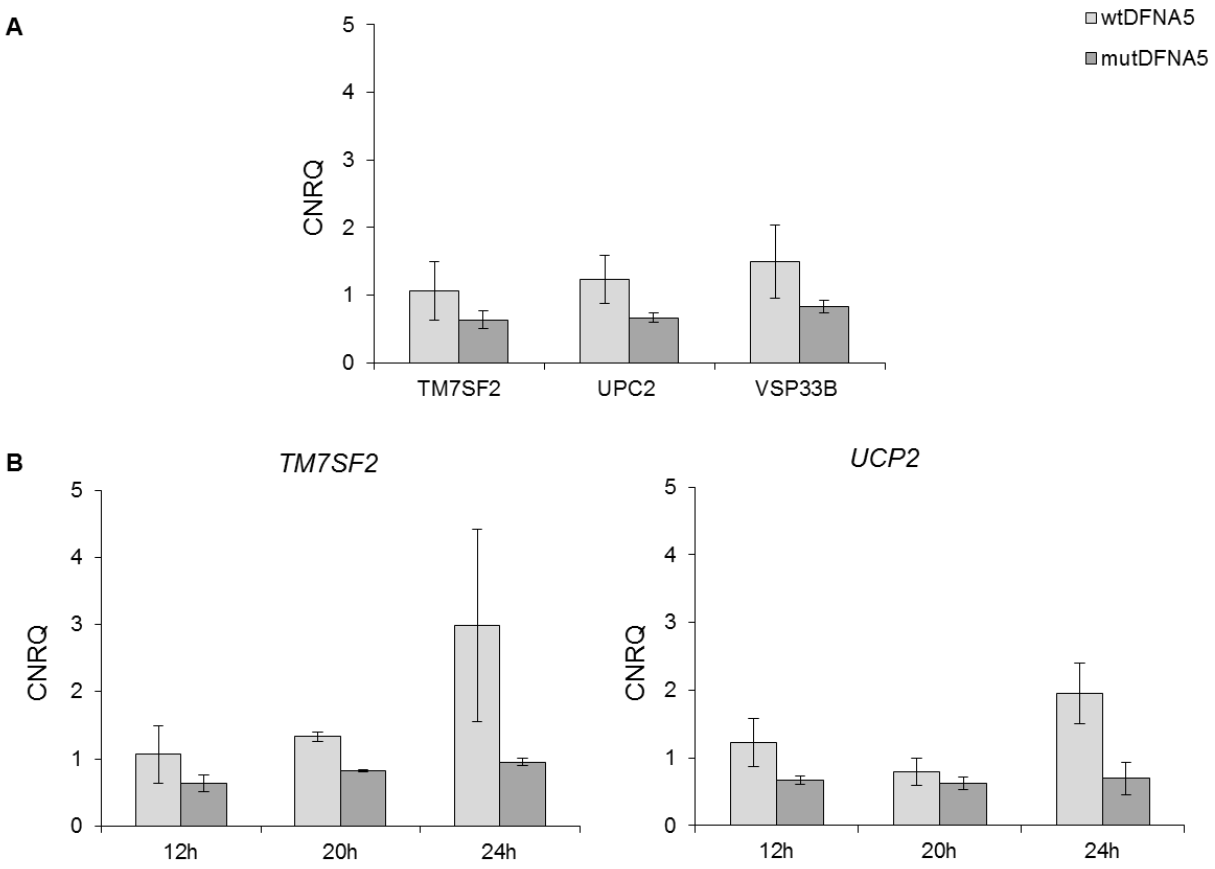

Fig. 5: Validation of the yeast microarray by real-time rtPCR in HEK293T cells. a. Gene expression 12 h post-transfection in human HEK293T cells of three selected genes. **b.** Gene expression 12 h, 20 h and 24 h post-transfection with either wt*DFNA5* or mutant *DFNA5*. *UCP2*, uncoupling protein 2 (mitochondrial, proton carrier); *TM7SF2*, transmembrane 7 superfamily member 2; *VPS33B*, vacuolar protein sorting 33 homologue. Light grey: wt*DFNA5*; Dark grey: mut*DFNA5*. *: $p < 0.05$.

3.5 Discussion

This study further investigated the biological differences induced by *DFNA5* in *Saccharomyces cerevisiae* and in human HEK293T cells. A previous study in human cell lines revealed that mut*DFNA5* is a cell death inducing gene [7]. As described in chapter II, transformation of mut*DFNA5* in yeast resulted in a growth defect associated with four different mitochondria-related proteins. This was the first time that a possible link between the mitochondria and DFNA5 was established. In addition, we observed that the protein quality control system, responsible for correct protein folding and degradation, had problems to cope with mutDFNA5.

This study confirmed the involvement of mitochondria-related processes upon expression of mut*DFNA5* in *Saccharomyces cerevisiae*, especially the ATP-coupled electron transport. Several genes related to either the glycolysis and the PPP were significantly down-regulated upon mut*DFNA5* transformation in yeast. Furthermore, we show that the JNK and ERK MAPK pathways are activated in vitro after transfection of mut*DFNA5* in HEK293T cells and that inhibition of this pathway is able to partially attenuate the resulting cell death. Additionally, this study also revealed an association of GO annotations related to the endoplasmic reticulum and protein folding in both model organisms.

3.5.1 Link to oxidative stress

The up-regulation of different cytochrome c oxidase (*COX*) genes in yeast revealed a potential association of the mutDFNA5-related cell death mechanisms with oxidative stress as enhanced COX activity has been associated with increased oxidative stress [20, 23]. Previous data indeed demonstrated a change in redox homeostasis due to mut*DFNA5* expression in yeast as described in chapter II. Here we showed increased oxidative stress measured by a dihydroethidium bromide staining (DHE). Moreover, preliminary experiments in human cell lines confirmed this and also revealed enhanced oxidative stress measured by a DHE staining (chapter IV). COX is the rate limiting enzyme of respiration which regulates the bio-energetic status of the cell. Dependent on the cellular energetic requirements, COX activity can be rapidly adapted. The ratio of ATP/ADP is

one of the regulators of the COX activity. High ADP levels or ATP utilisation will increase the enzyme activity and stimulate respiration [39, 40]. Activation of the cytochrome c oxidase activity could result in higher oxidative stress generated at the mitochondria and elicit mitochondria-related cell death.
Additionally, the correlation of mutDFNA5 with several genes of the PPP can also be linked to oxidative stress in yeast as the PPP plays a major role in the anti-oxidant response. Reduced expression of *CDC19*, the yeast homologue of PK, and of *TPI* has been correlated with the activation of the PPP and the redirection of the metabolic flux from glycolysis to PPP both in human cell lines and in yeast [24, 27, 41]. This study revealed both reduced gene expression of *TPI* and *CDC19* indicating a shift in redox sensing in eukaryotes mediating a fast response to oxidative stress. Activation of the PPP is correlated with the inhibition of ROS accumulation and enhancement of the anti-oxidant response upon shift from fermentation to respiration. PPP activation will enhance the anti-oxidant response and hence increase the tolerance for oxidative stress [26-28]. These data demonstrate a change in redox homeostasis due to mut*DFNA5* expression which was shown previously in yeast (chapter II).

Furthermore, enhanced oxidative stress is often related to a failing protein quality control system [42-46]. This possibility was already suggested by the proteolytic degradation previously seen upon wt*DFNA5* transformation in yeast, but which was absent in mut*DFNA5*-transformed yeast cells. MutDFNA5 seemed to escape this quality control system, in contrast to wtDFNA5 which was subject to the normal clearance system (see chapter II). This study thus already suggested a possible link between protein degradation and the mitochondria in mutDFNA5-induced cell death as yeast seemed to have problems with proper mutDFNA5 protein turnover and this link was confirmed in the current study.

3.5.2 Contribution of the mitochondria in MAPK-related cell death

In addition to the mitochondria, the MAPK pathways seem to play a prominent role in mutDFNA5-induced cell death in HEK293T cells. The link between the mitochondria and MAPK in DFNA5-related cell death is

unknown at this moment, but several mitochondria-MAPK correlations have been described. It is known that MAPK pathways can be induced by ROS production generated by the mitochondria [47]. As increased oxidative stress has been shown in an earlier study in yeast (see chapter II) and in human cell lines (described in chapter IV) this can provide a direct link between these two processes. This could suggest that ROS activates the MAPK pathway which places ROS up-stream of the MAPK pathway in DFNA5-induced cell death. However, a specific MAPK inhibitor was able to attenuate the cell death-inducing effects of mutDFNA5, shown in this study, and several anti-oxidants (data not shown) did not inhibit this process implying that ROS is either a secondary mutDFNA5-related event, or that the activation of MAPK is an early event in the cell death process situated up-stream of ROS production. Furthermore, comparison of yeast and the human microarray results identified *UCP2*, a gene associated with the mitochondria, which showed reduced gene expression in *mutDFNA5* transfected cells compared to *wtDFNA5* transfected cells. Interestingly, mitochondrial stress has been linked to the down-regulation of *UCP2* by activation of the MAPK pathway and of JNK activation in particular [48, 49]. *UCP2* reduction was an early event required for the amplification of the activated MAPK pathway enabling mitochondrial ROS production [49, 50]. This down-regulation enables mitochondrial ROS production providing the amplification loop stimulating the MAPK pathway.

UCP2 can therefore provide the link between MAPK and the mitochondria, regulating the ROS production, a feature dysregulated in both model organisms. The link between increased ROS, seen in HEK293T cells (described in chapter IV) and in yeast (chapter II), and the MAPK pathway is however, uncertain at this moment and needs to be further investigated.

3.5.3 Link ER and mitochondria

The increase in mitochondrial metabolism seen in this study, namely the ATP-coupled electron transport and respiration, and the decrease of protein folding processes, can also be explained by the presence of cellular ER stress leading to an unfolded protein response. Mitochondria and ER form an interconnected network which is important for several biological processes mediating an adaptive response under various cellular stress conditions [51,

52]. The association between mutDFNA5 and the GO terms related to lipid metabolism, protein targeting to ER and the ER membrane, can suggest the presence of ER stress [53]. Mitochondria depend on the ER for the import of several proteins and lipids and for Ca^{2+} exchange involved in cell death and mitochondrial metabolism [54-57]. Enhanced Ca^{2+} supply will increase ATP production and mitochondrial respiration, processes which were indeed both up-regulated at the post-diauxic shift upon mut*DFNA5* expression. Prolonged enhancement however will eventually have a detrimental effect on the mitochondria.

Despite the limited knowledge about the correlation of mutDFNA5 with ER stress, certain forms of HL have already been correlated with the presence of ER stress. The Usher syndrome for example, a syndromic form of hearing loss, is associated with enhanced ER stress due to protein trafficking defects upon mutations [58]. Other forms of HL, such as ototoxicity, were also shown to be correlated with ER stress-dependent pathways. Certain pharmaceutica lead to tinnitus and progressive bilateral sensorineural HL and showed increased ROS production, altered ER morphology and changes in ER stress markers, such as CHOP [59].

Taken together, the previous observation of the importance of the mitochondria in mutDFNA5-related cell death and the known correlation between the ER and the mitochondria underlines a potential role for the ER in DFNA5-induced cell death. The failing of the protein quality control system in mutDFNA5, described in chapter II, suggests the involvement of the ER, but remains unclear at this moment and needs to be further investigated in the future.

In conclusion, this study confirms the role of the mitochondria in mutDFNA5-induced toxicity in yeast. Additionally, it showed that mutDFNA5-induced cell death is mediated by the MAPK pathway, especially through the ERK and the JNK branch. Inhibition of this pathway could significantly enhance the cell viability of mut*DFNA5*-transfected HEK293T cells which suggests the importance of this signalling cascade for DFNA5. How the MAPK pathways perform their cell death role remains uncertain at this moment. The importance of the ER needs to be further investigated in

the future. The connection with DFNA5-related mechanism is unclear at this moment, but is a good starting point for future studies to unravel the DFNA5-induced cell death mechanism which can lead to new therapies and to new insights in HL-related to mitochondrial dysfunction.

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Chapter IV

Mutant DFNA5-induced cell death is mediated by mitochondrial dysfunction

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Work in progress

All the DFNA5 plasmid constructs used in this chapter were made by Dr. Ken Op de Beeck. The caspase and the LC3 expression experiments were performed in collaboration with respectively the lab of Prof. Vandenabeele and Prof. Martinet. The rest of the scientific experiments were performed by Sofie Van Rossom. All the co-authors contributed to the corrections and revision of the written report.

4 Mutant DFNA5-induced cell death is mediated by mitochondrial dysfunction

4.1 Abstract

Programmed cell death (PCD) is widely present, tightly regulated and essential for survival. Several forms of PCD exist side by side and these are often highly interconnected. Hearing loss (HL) is one specific pathology which has been linked to PCD, especially in the development of the vertebrate inner ear and in the morphogenesis of the semicircular canals [1]. Apoptosis has been specifically linked to the degeneration of the auditory sensory hair cells. Mice deficient in caspase-3, a marker for apoptosis, show elevated ABR thresholds and hair cell loss at day 30 [2]. Similarly, oxidative stress, another cell death hallmark, can damage the auditory sensory hair cells in rats shown by Kopke et al [3]. Due to its prominent role in hearing impairment, several types of HL have been linked to PCD, such as age-related hearing impairment (ARHI), noise-induced hearing loss (NIHL) and monogenic HL. Noise-induced damage of hair cells for example could be prevented by the inhibition of apoptosis [4]. This study will focus on PCD-related to *DFNA5*, a gene responsible for autosomal non-syndromic dominant HL. Mutations in *DFNA5* leading to exon 8 skipping, result in HL in several families. In addition to HL, *DFNA5* was identified as a tumour suppressor gene involved in different forms of cancer, like breast, colorectal, gastric and melanoma cancer.

We characterised mutant *DFNA5* as a cell death-inducing gene both in human cell lines [5] and in *Saccharomyces cerevisiae*. Moreover, several mitochondrial proteins, like Fis1, Por1, Aac1 and Aac3, were identified contributing to mutant DFNA5-related cell death in yeast (chapter II). These four proteins were all related to mitochondrial fragmentation or permeabilisation indicating a correlation of mutant DFNA5 with the mitochondria.

In order to further elucidate the cell death mechanisms related to HL, we performed several mitochondrial assays to investigate their role in the induction of apoptotic cell death in human cell lines. We showed that

dysfunctional mitochondria are clearly present in mutant *DFNA5*-transfected HEK293T cells, as demonstrated by the release of cytochrome c and citrate synthase, loss of the mitochondrial membrane potential and increased oxidative stress. Additionally, accumulation of damaged mitochondria, as shown by disintegration of the mitochondrial network, was present upon mutant *DFNA5* transfection, although their association with mutant DFNA5 is still unclear.

4.2 Introduction

Cell death is inherent to life and essential during numerous cellular processes, such as development, organogenesis and the function of the immune system. Excessive or scarce cell death however, has major implications on tissue homeostasis [1, 4]. It is essential to maintain this delicate task in balance and therefore cell death is tightly regulated.

As described in chapter II we characterised mutant *DFNA5* as a cell death-inducing gene in human cell lines and in *Saccharomyces cerevisiae*. Moreover, several mitochondrial proteins, like Fis1, Por1, Aac1 and Aac3, were identified in yeast contributing to mutant DFNA5-related cell death. In order to further elucidate the cell death mechanisms related to HL, we performed several mitochondrial assays to investigate their role in the DFNA5-induced cell death in human cell lines. Former results characterised DFNA5-induced cell death as apoptotic based on TUNEL positivity and a AnnexinV/PI staining [5]. Re-evaluation of the former results obtained in human cell lines displayed some uncertainties concerning the role of apoptosis. Therefore, we started with the investigation of the role of caspases and the release of cytochrome c, typical hallmarks of apoptosis. Additionally, two caspase-independent forms of PCD were evaluated, namely necroptosis and ferroptosis.
Besides apoptosis, mitochondria could also play a central role during autophagy, more in particular in mitophagy. Therefore, some preliminary autophagic experiments were performed in order to unravel the role of this survival pathway in DFNA5-induced cell death.

Necroptosis, ferroptosis and autophagy were inhibited by respectively Nec-1s, erastin and 3-methyladenine (3-MA) to determine their role in mut*DFNA5*-transfected HEK293T cells. No specific form of cell death could be attributed to mutDFNA5, but transfection of HEK293T cells with mut*DFNA5* resulted in the disintegration of the mitochondrial network, the release of cytochrome c and the matrix citrate synthase suggesting mitochondrial collapse. Accumulation of damaged mitochondria is thus inherent to mutDFNA5-induced cell death, but the link between the mitochondria and mutDFNA5-associated cell death remains unknown.

4.3 Material and methods

4.3.1 Reagents

Ferrostatin-1 (SML0583) and 3-methyladenine (3-MA, M9281) were purchased from Sigma-Aldrich (MO, USA). Z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk, N-1510.0005) was purchased from Bioconnect Lifesciences (Huissen, Nederland). Nec-1s was kindly provided by Prof. Koen Augustyns (Laboratory of Medicinal Chemistry, University of Antwerp). Necrox and t-BHP (tert-butylhydroperoxide) were kindly provided by Prof. Wim Martinet (Laboratory of Physiopharmacology, University of Antwerp). Erastin was kindly provided by Prof. Peter Vandenabeele (VIB Inflammation Research Center, University of Gent).

4.3.2 Constructs, cell culture and growth conditions

Human full-length *DFNA5* cDNA constructs, previously described by Gregan *et al.*, were used as a template to amplify wt*DFNA5* and mut*DFNA5* [5, 6]. pEGFP-N1 was either used as the ligation vector for the constructs or left empty to use as a control (EGFP, hereafter referred to as control). All constructs were verified by bidirectional sequencing on an ABI genetic analyser 3130xl (Applied Biosystems, Foster City, CA, USA).

Human Embryonic Kidney 293T cells (HEK293T) were subcultured in a 6-well plate at a density of 8 x 10^5 cells in Dulbecco's modified Eagle's medium containing 4500 mg/l glucose supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine ($DMEM^+$). Overnight cultures were transfected with control, wt*DFNA5* or

mut*DFNA5* using lipofectamine. Six hour after incubation with serum-free Optimem, cells were incubated in DMEM^{+} medium. Cells were harvested using Triple-x reagent. All products for human cell cultures were purchased from Invitrogen (San Diego, USA).

4.3.3 Western Blot analysis

For western blotting, cells were lysed using RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Pierce, Rockford, IL, USA) containing a PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche), an EDTA-free protease tablet and 10 µl (25units/µl) benzonase (70746-4 Novagen®, Merck Millipore, Darmstadt, Germany). Transfected HEK293T cells were lysed for 20 minutes at 4°C and centrifuged at 2000 *g* at 4°C to obtain protein lysates.

Proteins were electrophoretically separated and blotted onto a nitrocellulose membrane (Whatman, Kent, UK). This membrane was blocked for an hour in 5% non-fat dry milk and incubated overnight (4°C) in one of the following primary antibodies: anti-LC3 (5F10 clone, NanoTools, Teningen, Germany), anti-cleaved caspase-3 (#9661), anti-cleaved caspase-8 (#9496S), anti-caspase-9 (#9508) (Cell Signalling Technologies, MA, USA), anti-citrate synthase (sc-390693), anti-cytochrome c (K265-100, Biovision, CA, USA) or anti-β-Actin (A5316, Sigma Aldrich, MO, USA). After washing, the membranes were incubated with either a secondary goat anti-rabbit (ab6721, Abcam, Cambridge, UK) or sheep anti-mouse (NA931, GE Healthcare, Buckingmersham, UK) antibody. Finally, the corresponding proteins were visualised using Enhanced ChemiLuminescence Western Blotting Substrate (Thermo Scientific, IL, USA). Quantification of the protein levels was performed using the ImageQuant TL software (GE Healthcare). The LC3 protein expression was visualised by InfraRed (700 nm) detection using an Odyssey Sa Infrared imaging system (Li-Cor, NE, USA).

4.3.4 DNA laddering

DNA fragmentation was measured using the Apoptotic DNA Ladder Kit from Roche (Cat:11 835 246 001, Basel, Switzerland) and performed according to the manufactures instructions.

4.3.5 Mitochondrial assays

4.3.5.1 Mitochondrial membrane potential and oxidative stress

Tetra Methyl Rhodamine Methylester (TMRM, Sigma Aldrich, MO, USA) was used to measure the mitochondrial membrane potential (Δψ). TMRM is a mitochondrial staining for which uptake is dependent on the presence of the Δψ. Twelve hours post-transfection, HEK293T cells were incubated with 200 nM TMRM for 10 minutes at 37°C.

Oxidative stress was measured by incubating HEK293T cells, 12 h post-transfection, for 10 minutes with 2.5 µg/ml dihydroethidium (DHE, Sigma Aldrich, MO, USA) at room temperature. After these stainings, cells were collected and measured by flow cytometry (CyFlow ML, Partec, Germany).

4.3.5.2 Cytochrome c and citrate synthase detection

To measure the release of cytochrome c and citrate synthase to the cytosol, transfected HEK293T cells were fractionated to obtain a mitochondrial and a cytosolic protein fraction. HEK293T cells were gently lysed for 15' on ice using a HEPES buffer (0.05 nM HEPES, 150 mM NaCl, pH 7.4) containing 25 µg/ml digitonin (Invitrogen, San Diego, USA). A first centrifugation step (200*g*, 4°C) retrieved the supernatants containing the cytosolic fraction. The pellet was further lysed following the protocol described above (4.3.3.). A cytosolic (GAPDH, #2118, Cell Signalling, MA, USA) and a mitochondrial (VDAC, #4866, Cell Signalling, MA, USA) protein was analysed to determine the purity of each fraction by western blotting.

4.3.5.3 Fluorescence microscopy

To investigate the integrity of the mitochondrial network, mitochondria were stained with a DsRed fluorescent protein fused to a mitochondrial targeting signal (MTS). An MTS sequence (ATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGCTCGGCCCGGCGG CTCCCAGTGCCGCGCGCCAAGATCCATTCGTTG) was cloned and fused into a DsRed ligation vector (DsRed monomer N1 Clontech). This construct was verified by bidirectional sequencing on an ABI genetic analyser 3130xl (Applied Biosystems, Foster City, CA, USA). HEK293T cells were transfected

with either wt*DFNA5* or mut*DFNA5* and DsRed-MTS plasmid to visualise the mitochondria.

4.3.6 Statistical analysis

Each experiment was performed in triplicate and statistical analyses were performed by the SPSS software (SPPS Statistics 20, Inc. Chicago, IL, USA) using either one- or two-way ANOVA. The statistical significance level was set to 0.05.

4.4 Results

4.4.1 Mutant DFNA5 induces cell death in HEK293T cells

In order to further investigate the growth defect induced by mutDFNA5, we performed a preliminary cell viability experiment by flow cytometry to determine the amount of cell death. As shown in Fig. 1a, mutDFNA5 indeed induced a significant growth defect ($p<0.001$) in HEK293T cells compared to wtDFNA5 and the control. Cell viability decreased from 89.36% and 86.00%, in respectively wt*DFNA5* and control cells, to 22.33% in mut*DFNA5*-transfected HEK293T cells.

Previously published research in HEK293T cells specified the type of cell death as apoptotic, as determined by a TUNEL and an AnnexinV/PI staining [5]. However, re-evaluation of the former results prompted us to investigate this further before drawing final conclusions. To further determine the type of cell death, we performed a DNA electrophoresis experiment to study the DNA laddering pattern, typical for apoptotic cells. DNA was extracted using the Apoptotic DNA Ladder Kit (Roche, Basel, Switzerland) and as shown in Fig. 1b no DNA laddering pattern was seen in mut*DFNA5*-transfected HEK293T cells when compared to the positive control. A smear of DNA was visible when only non-adherent mut*DFNA5*-transfected HEK293T cells were studied indicating random cleavage of DNA contrasting the typical, non-random DNA laddering pattern of apoptotic cells. This result indicated that more research is needed to unravel the true nature of mutDFNA5-induced cell death.

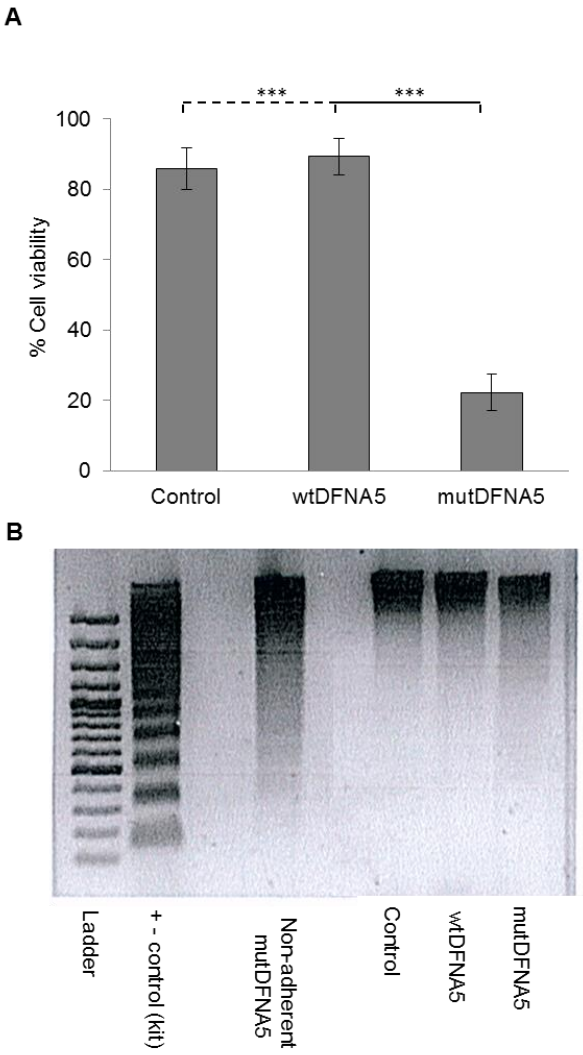

Fig. 1: MutDFNA5 induces cell death in HEK293T cells. a. Cell viability of HEK293T cells transfected with a control plasmid, wt*DFNA5* or mut*DFNA5*. Viability was measured the following day by flow cytometry using PI as a cell death marker. Only GFP positive cells were taken into account and at least 10000 events were recorded each time. **b.** DNA laddering pattern of control, wt*DFNA5*-and mut*DFNA5*-transfected HEK293T cells. 'Non-adherent mutDFNA5' indicates cells which were no longer adherent to the cell culture dish and represent dead cells. * denotes a significant difference in cell viability of mutDFNA5 compared to control and wtDFNA5. ***: $p<0.001$.

4.4.2 MutDFNA5-induced cell death is caspase and RIPK1 independent

Due to the discrepancies between the former TUNEL staining and the DNA laddering experiment, we started with some basic experiments to re-evaluate the exact type of cell death induced by mutDFNA5. To further determine the role of apoptosis, cleavage of several caspases was evaluated by western blot as these are the key mediators of apoptosis and can differentiate between apoptosis and other forms of PCD. As overexpression of RIPK1, a kinase involved in necroptosis and apoptosis, is known to induce caspase cleavage through the extrinsic apoptotic pathway in human cell lines, *RIPK1*-transfected HEK293T cells were used as a positive control. Overexpression of RIPK1 is able to induce caspase-dependent cell death by recruiting caspase-8. *MLKL*-transfected HEK293T cells, another key player of necroptosis, were used as a negative control as MLKL induces cell death that is caspase-independent.

Before protein lysates were taken to study caspase cleavage, the presence of cell death was measured by SytoxGreen, a cell impermeable dye, to ascertain the induction of cell death (Fig. 2a). Mut*DFNA5*-transfected cells indeed showed an increase in the amount of cell death compared to control, wt*DFNA5*- and *RIPK1*-transfected HEK293T cells. Next, protein lysates were taken and caspase cleavage was measured. No cleavage of caspase-3 nor caspase-8 was seen in mut*DFNA5*-transfected HEK293T cells when compared to the *RIPK1*-transfected HEK293T cells (Fig. 2b). No caspase-9 cleavage was seen in any of the samples (data not shown).

Due to the lack of caspase cleavage, the classical apoptotic pathway could be excluded. These results demonstrate that a clear difference exists between the mechanism induced by mutDFNA5 and by RIPK1 in HEK293T cells.

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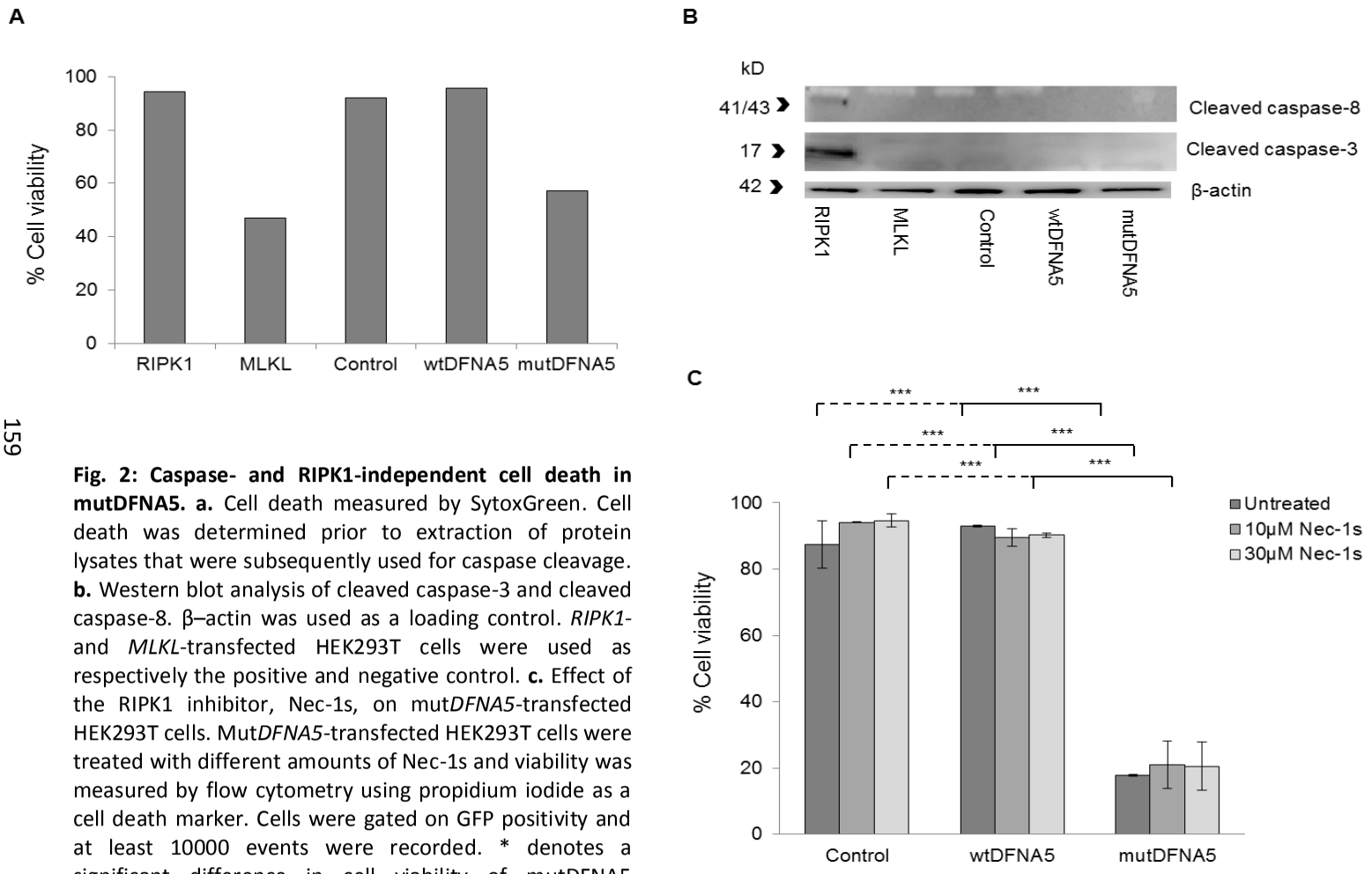

Fig. 2: Caspase- and RIPK1-independent cell death in mutDFNA5. a. Cell death measured by SytoxGreen. Cell death was determined prior to extraction of protein lysates that were subsequently used for caspase cleavage. **b.** Western blot analysis of cleaved caspase-3 and cleaved caspase-8. β–actin was used as a loading control. *RIPK1*- and *MLKL*-transfected HEK293T cells were used as respectively the positive and negative control. **c.** Effect of the RIPK1 inhibitor, Nec-1s, on mut*DFNA5*-transfected HEK293T cells. Mut*DFNA5*-transfected HEK293T cells were treated with different amounts of Nec-1s and viability was measured by flow cytometry using propidium iodide as a cell death marker. Cells were gated on GFP positivity and at least 10000 events were recorded. * denotes a significant difference in cell viability of mutDFNA5 compared to control and wtDFNA5. ***: $p<0.001$.

This was also confirmed when the role of the kinase domain of RIPK1 was studied. The kinase domain of RIPK1 and its co-factor RIPK3, a necroptotic component is missing in HEK293T cells, are necessary to induce necroptosis. This kinase domain can specifically be inhibited by Nec-1s, a stable form of necrostatin. Addition of Nec-1s however, does not ameliorate the viability of mut*DFNA5*-transfected HEK293T cells as shown in Fig. 2c. Different concentrations of Nec-1s were used and transfected HEK293T cells were incubated overnight. Neither 10 µM nor 30 µM Nec-1s could increase the cell viability of mut*DFNA5*-transfected HEK293T cells compared to control and wt*DFNA5*, confirming the independency of RIPK1 for mutDFNA5-associated cell death. No significant decrease in cell viability could be detected when treated and untreated, mut*DFNA5*-transfected HEK293T cells were compared (Fig. 2c).

The difference in type of cell death between RIPK1 and mutDFNA5 could already be assumed by the cell viability experiment shown previously (Fig. 2a). this demonstrated enhanced cell death in mut*DFNA5*-transfected HEK293T cells, but not in *RIPK1*-transfected cells indicating a different mechanism compared to mutDFNA5. Based on these results, we conclude that mutDFNA5-associated cell death is independent of caspases and RIPK1 which excludes necroptosis and the classical apoptotic pathway.

4.4.3 Mutant DFNA5 expression leads to accumulation of damaged mitochondria

Research in yeast identified several proteins, such as Fis1, Aac1, Aac3 and Por1, related to either mitochondrial fragmentation or permeabilisation (see chapter II). Therefore we wondered whether mutDFNA5 induces cell death through a mitochondrial pathway in human cell lines. This led us to investigate the mitochondrial morphology by fluorescence microscopy to determine whether the mitochondrial network is still intact. A mitochondrial targeting signal (MTS) was cloned into a DsRed ligation vector and this *DsRed-MTS* construct was transfected in HEK293T cells with wt*DFNA5* or mut*DFNA5*. HEK293T cells transfected with *DsRed-MTS* alone were used as a control. As demonstrated in Fig. 3, wt*DFNA5*-transfected HEK293T cells had an intact mitochondrial network. The presence of tubular mitochondrial structures under these conditions clearly demonstrates their integrity. In

mut*DFNA5*-transfected HEK293T cells on the other hand, punctuate mitochondrial structures were visible and no tubular mitochondria were present, suggesting altered mitochondrial morphology. These results demonstrate the accumulation of damaged mitochondria induced by mutDFNA5. As damaged mitochondria were only seen in mutDFNA5 cells, disintegration of the mitochondrial network is probably induced by mutDFNA5. How mutDFNA5 regulates this process remains unknown at this moment. However, no co-localisation of mutDFNA5 and the mitochondria was seen (Fig. 3 overlay) leading us to conclude that this interference is probably indirect.

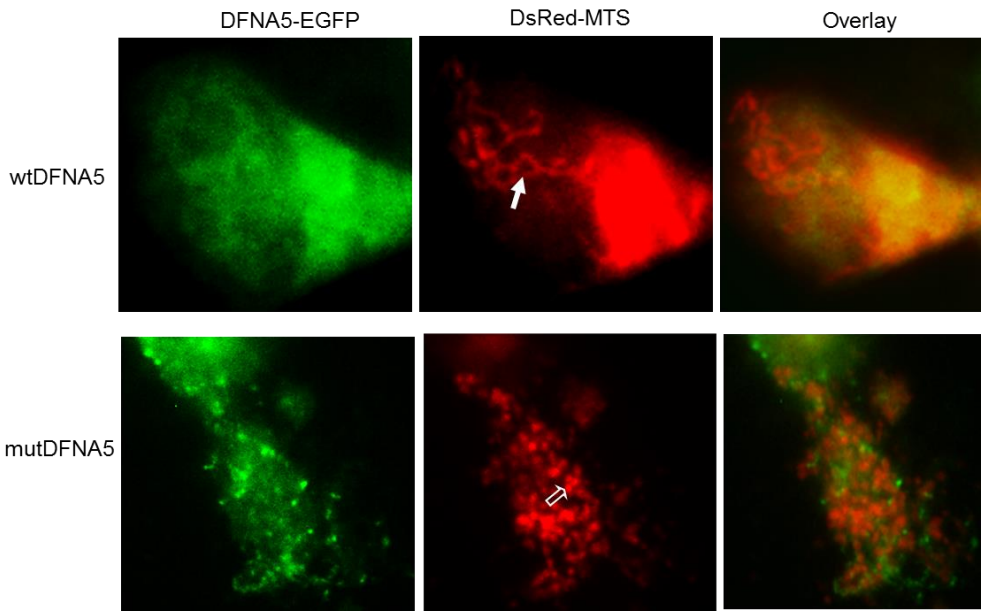

Fig. 3: Visualisation of the mitochondrial network. Fluorescence microscopy of HEK293T cells transfected with DsRed plasmid fused to a MTS and wt*DFNA5* or mut*DFNA5*. DsRed-MTS and wt*DFNA5*-transfected HEK293T cells show an intact mitochondrial network containing tubular structures (filled white arrow), whereas in mutDFNA5 cells dysfunctional mitochondria are present as shown by the presence of punctuate mitochondria (open white arrow). The expression of DFNA5 is shown in green, the mitochondria in red. MTS, mitochondrial targeting signal.

4.4.4 MutDFNA5 induces mitochondrial alterations in HEK293T cells

Due to the loss of an intact mitochondrial network, several mitochondrial assays were performed to investigate the functionality of the mitochondria. We first investigated the amount of oxidative stress. Mitochondria are the major source of oxidative stress due to the mitochondrial process of oxidative phosphorylation which generates free radicals. ROS was measured by DHE staining 12 h post-transfection. A significant difference in the amount of oxidative stress was seen in *DFNA5*-transfected HEK293T. A significant increase in oxidative stress from 4.59% to 28.67% DHE positivity was observed upon mut*DFNA5* transfection compared to wt*DFNA5* ($p<0.01$) suggesting oxidative stress mediated by mutDFNA5 (Fig. 4a).

Next, the mitochondrial membrane potential (ΔΨ) was measured to further investigate the role of the mitochondria in mutDFNA5-induced cell death. Loss of ΔΨ could suggest opening of the permeability transition pore. To measure ΔΨ, HEK293T cells were stained with TMRM, 12 h post-transfection, and measured by flow cytometry. Cells were gated on GFP positivity and viability and at least 10000 events were recorded. A reduction in TMRM fluorescence was observed upon mut*DFNA5* transfection indicating decreased ΔΨ supporting mitochondrial permeabilisation (Fig. 4b).

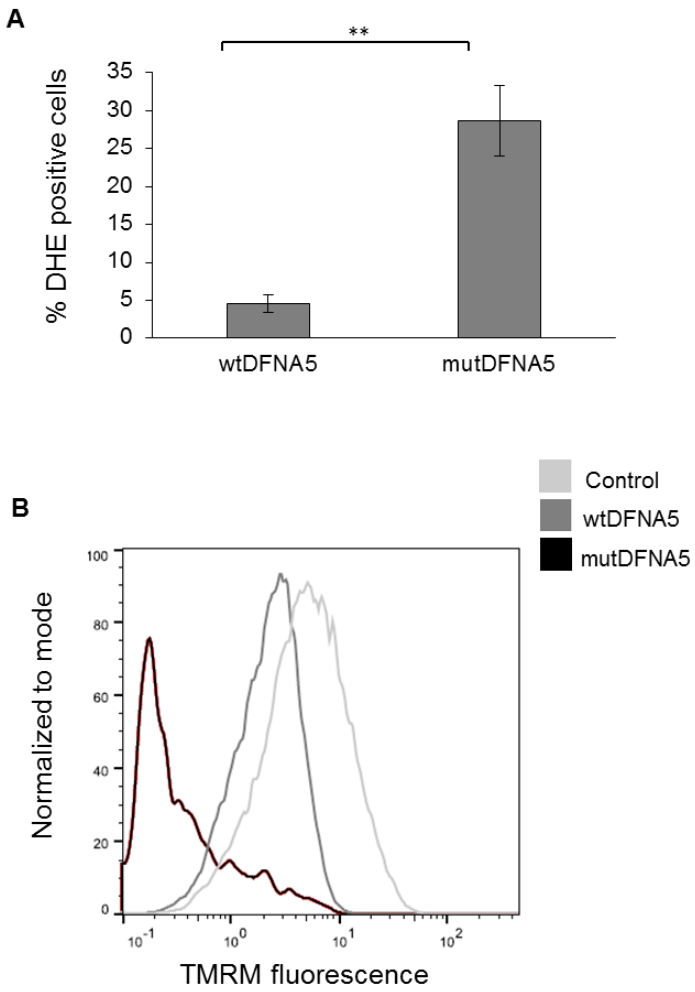

Fig. 4: Mitochondrial alterations induced by mutDFNA5. a. DHE staining measuring oxidative stress. Transfected HEK293T cells were stained with 2.5 µg/ml dihydroethdium (DHE) and measured by flow cytometry. Significant increase in oxidative stress was seen in mutDFNA5 HEK293T cells compared to wtDFNA5. Cells were gated on GFP positivity and at least 10000 events were recorded. **b.** TMRM assay measuring the mitochondrial membrane potential (Δψ). Significant decrease of the Δψ was seen upon transfection of mut*DFNA5*. Cells were gated on GFP positivity (transfected HEK293T cells) and on viability. 'Normalised to mode' means that the % of maximum was taken. This is the # of cells in each bin divided by the # of cells in the bin that contains the largest number of cells. **: $p<0.01$.

Release of cytochrome c and citrate synthase to the cytosol

The partial loss of ΔΨ can initiate the release of several apoptotic factors by mitochondrial permeabilisation. One of the main factors to be released through mitochondria is cytochrome c. Under normal conditions, cytochrome c resides in the mitochondria, but it is released to the cytosol upon induction of apoptosis. To verify the cellular location of cytochrome c, fractionated protein lysates were collected at different time-points post-transfection obtaining a cytosolic and mitochondrial protein fraction. The presence of cytosolic cytochrome c was determined by western blot using a specific cytochrome c antibody. First, the integrity of the mitochondrial preparation during fractionation was verified to determine the purity of each fraction. Cytosolic GAPDH and mitochondrial VDAC antibodies were used as controls for respectively the mitochondria and the cytosol. No VDAC and GAPDH was present in respectively the cytosolic and the mitochondrial protein lysate, demonstrating a correct fractionation procedure obtaining pure protein fractions (Fig. 5a). Therefore, cytosolic cytochrome c would support mitochondrial permeabilisation. No increase in cytosolic cytochrome c release was seen in mut*DFNA5*-transfected HEK293T cells at 9 h, 12 h and 16 h post-transfection (data not shown), but as shown in Fig. 5b, increased release of cytochrome c to the cytosol was indeed seen at 20 h post-transfection. Mut*DFNA5*-transfected HEK293T cells showed elevated cytochrome c levels compared to control and wt*DFNA5*. Quantification of the cytochrome c protein levels in relation to GAPDH, the loading control, confirmed the significant increase of cytosolic cytochrome c release ($p(<0.001)$ and $p(<0.001)$ compared to respectively control and wtDFNA5) (Fig. 5b). This release was six times higher in mutDFNA5 protein samples compared to control and wtDFNA5. Cytochrome c however, partially remained in the mitochondria suggesting restricted mitochondrial permeabilisation (Fig. 5b).

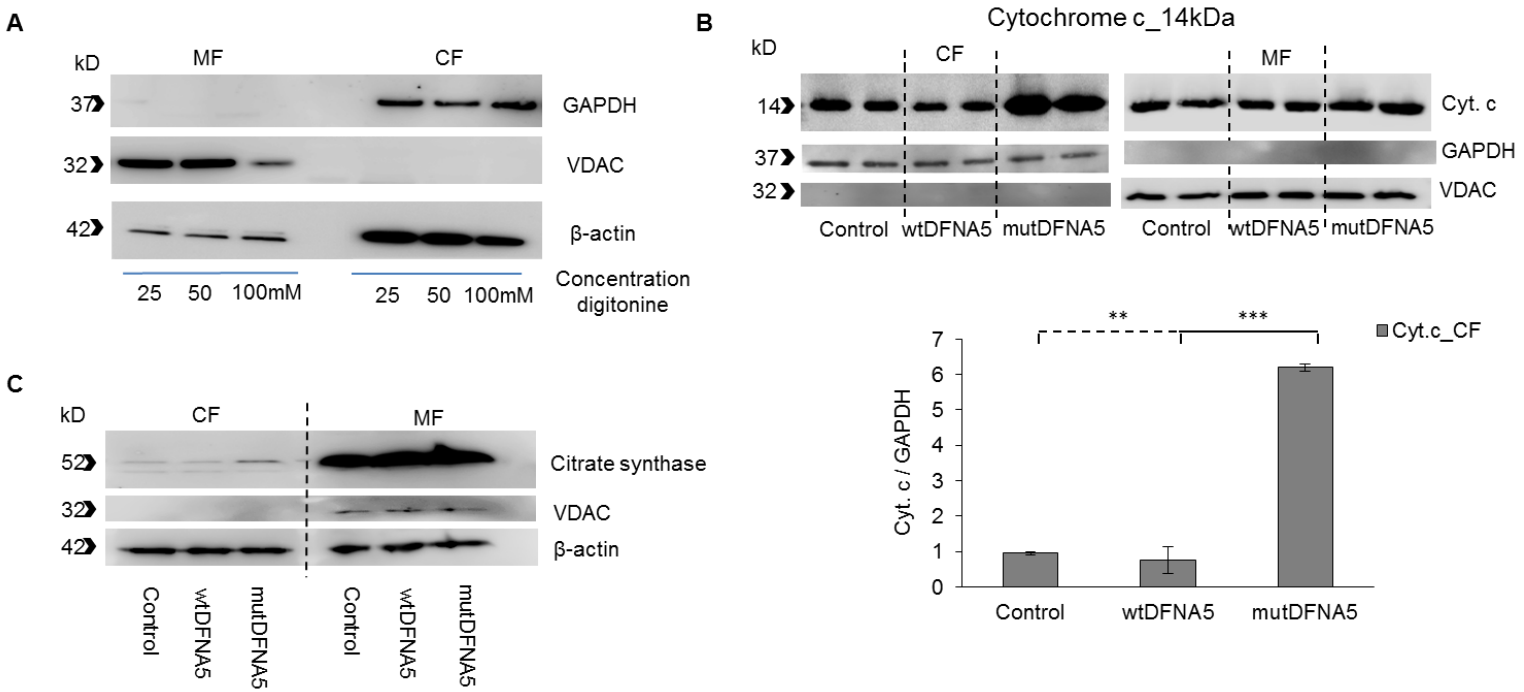

Fig. 5: Western blot analysis of cytochrome c and citrate synthase expression in the cytosolic and mitochondrial fraction. a. Different concentrations of digitonin were used to optimise the fractionation procedure in order to obtain pure protein fractions. No VDAC or GAPDH expression could be observed in respectively the cytosolic or mitochondrial fraction. The decrease of VDAC in the mitochondria could indicate some release to the cytosol. Therefore, a concentration of 25 mM was chosen to ascertain the purity of the cytosolic protein fraction in order to correctly quantify the release of cytochrome c to the cytosol. **b.** Western blot analysis of cytochrome c in the cytosolic fraction 20 h post-transfection and quantification (below) of the cytochrome expression in relation to GAPDH. Increased release was seen in mutDFNA5 samples compared to control and wtDFNA5. **c.** Western blot analysis of citrate synthase in the cytosolic fraction 20 h post-transfection. Increased release was seen in mutDFNA5 samples compared to control and wtDFNA5. * denotes a significant difference of cytochrome c expression in mutDFNA5 compared to control and wtDFNA5. **: $p<0.01$; ***: $p<0.001$; CF, cytosolic fraction; MF, mitochondrial fraction; cyt.c, cytochrome c.

Although cytochrome c was released to the mitochondria, no caspase cleavage could be detected. Therefore, the mitochondrial integrity was further determined by investigating the release of citrate synthase. Citrate synthase, a mitochondrial matrix protein, is often used as a marker for intact mitochondria as it is retained in the mitochondria during permeabilisation. In addition to the release of cytochrome c, 20 h post-transfection increased release of citrate synthase to the cytosol was seen in HEK293T cells transfected with mut*DFNA5* compared to control and wt*DFNA5* (Fig. 5c). Citrate synthase was still detectable in the mitochondrial fraction, suggesting partial disintegration of the mitochondria. This is similar to cytochrome c which also partially remained in the mitochondria. Release of citrate synthase however, clearly demonstrates loss of intact mitochondria. As the VDAC protein was only present in the mitochondrial fraction, the release of citrate synthase could not be attributed to an erroneous fractionation procedure. As increased release of citrate synthase to the cytosol was seen in mutDFNA5 compared to control and wtDFNA5, this release is probably induced by mutDFNA5.

The increase in oxidative stress, the loss of the mitochondrial membrane potential and the release of cytochrome c and citrate synthase indeed confirm the presence of damaged mitochondria induced by mutDFNA5. Due to the lack of caspase cleavage, the release of cytochrome c could be a secondary event in the cell death pathway. This release can be due to damaged mitochondria, explaining the absence of caspase activation.

4.4.5 MutDFNA5-induced cell death is independent of Necrox or ferrostatin

The presence of damaged mitochondria indicates a role for the mitochondria in mutDFNA5-induced cell death. Besides apoptosis, other forms of PCD are related to the mitochondria and characterised by enhanced ROS production. Therefore, we tested two hypotheses to study the role of ROS in the caspase-independent mutDFNA5-induced cell death.
We wondered whether the increased oxidative stress is the result of ferroptosis, a specific non-apoptotic, iron-dependent form of cell death characterised by enhanced ROS [7]. Furthermore, we also investigated whether the oxidative stress could be alleviated by Necrox, a novel class of mitochondrial ROS scavengers involved in non-apoptotic cell death [8, 9].

Different concentrations of Necrox were used and their effect on cell viability of HEK293T-transfected cell lines was studied. As a positive control for mitochondrial ROS production, 500 µM t-BHP, a pro-oxidant compound, was used whose toxic effects could be abolished by Necrox. Mut*DFNA5*-transfected or untransfected HEK293T cells were incubated overnight with different concentrations of Necrox. Pretreated HEK293T cells were incubated, 16 h post-transfection, with t-BHP for 4 hours as a positive control for Necrox. As shown in Fig. 6, addition of 10 µM Necrox did not have any major influence on the amount of cell death induced by mut*DFNA5* when compared to untransfected HEK293T cells. Addition of 500 µM t-BHP for 4 hours decreased cell viability which could be partially inhibited by overnight pretreatment of HEK293T cells with 10 µM Necrox, indicating that Necrox is indeed taken up by the HEK293T cells (Fig. 6a). Addition of higher Necrox concentrations like 20 µM or 50 µM did also not alter the cell viability of mut*DFNA5*-transfected HEK293T cells (Suppl Fig. 4.1a.).

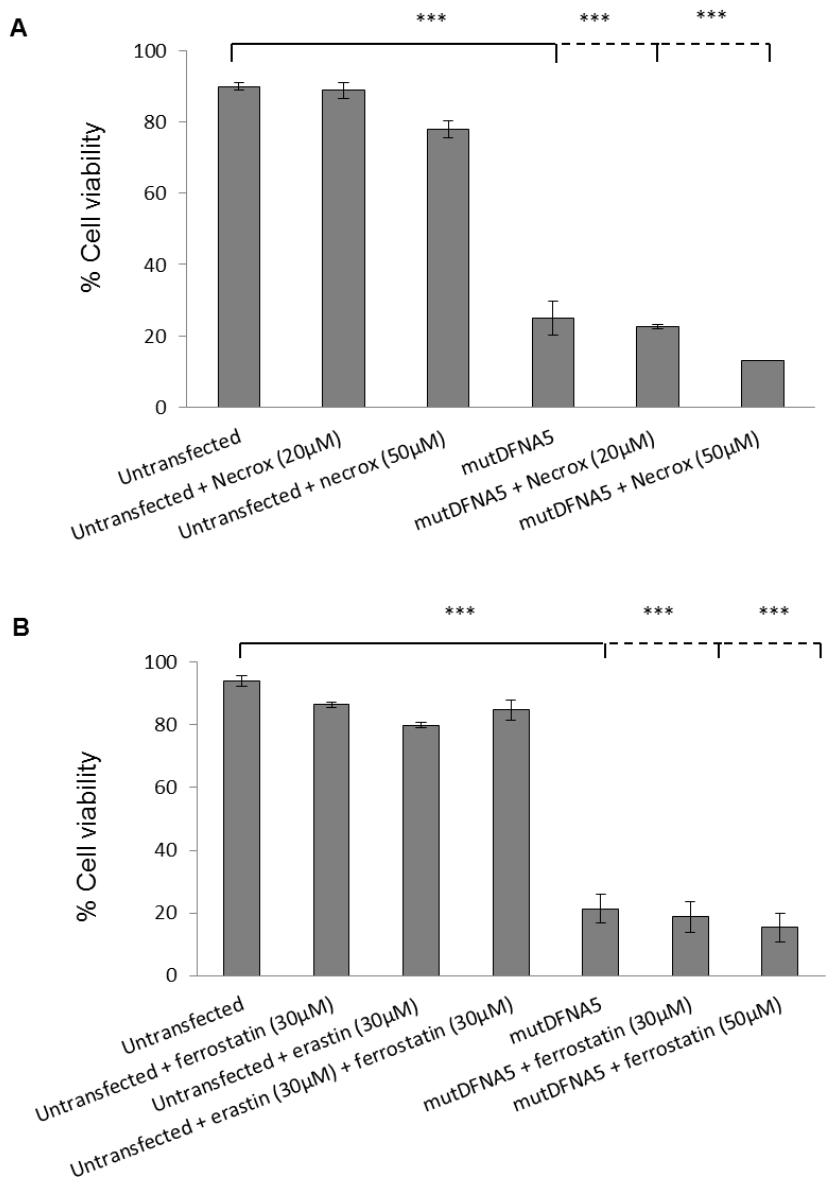

Fig. 6: Role of Necrox and ferrostatin on mutDFNA5 cell viability. a. 10 µM Necrox was added overnight for 16 h to mut*DFNA*5-transfected and untransfected HEK293T cells. As a positive control, pretreated HEK293T cells were incubated for 4 hours with t-BHP. Cell viability was measured by flow cytometry using PI as a cell death marker. **b.** 10 µM ferrostatin and/or erastin was added overnight for 16 h to mut*DFNA5*-transfected and untransfected HEK293T cells. Cell viability was measured the following day by flow cytometry using PI as a cell death marker. * denotes a significant difference in cell viability in mut*DFNA5* compared to untransfected HEK293T cells. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. t-BHP, tert-butylhydroperoxide.

As the cell viability could not be ameliorated by Necrox, a specific mitochondrial ROS scavenger, we wondered whether ferrostatin, a specific inhibitor of ferroptosis, could decrease cell death induced by mutDFNA5. Ferrostatin-1, a ROS scavenger preventing damage to membrane lipids, can inhibit ferroptosis, indicating that lipid peroxidation contributes directly to this type of cell death by production of lipid ROS [7, 10]. Erastin was used as a positive control for ferroptosis induction. Erastin inhibits the cystin/glutamate receptor leading to depletion of glutathion and increase of ROS.
Transfected HEK293T cells were incubated overnight with concentrations of ferrostatin and/or erastin and cell viability was measured the following morning to determine their role in mutDFNA5-induced cell death. As demonstrated in Fig. 6b, addition of 10 µM ferrostatin could not increase cell viability of mut*DFNA5*-transfected HEK293T cells indicating that ferroptosis does not play a major role in DFNA5-associated cell death. Addition of higher ferrostatin concentrations like 30 µM or 50 µM ferrostatin did also not alter the cell viability of mut*DFNA5*-transfected HEK293T cells (Suppl Fig. 4.1b).

The ferrostatin inducer erastin however, did not decrease cell viability of untransfected HEK293T cells. This could be due to the fact that erastin was originally identified as a component selectively lethal to RAS-mutant cell lines and that HEK293T cell lines do not contain a mutation in the RAS oncogene [7].

4.4.6 Autophagy

As no specific type of cell death could be linked to mutDFNA5, therefore we focused on autophagy, an important, pro-survival mechanism maintaining cellular homeostasis. Autophagy can function as a redox sensor and can be preceded by a decrease in mitochondrial membrane potential. Moreover, mitochondrial damage, can suggest mitophagy, a specific autophagic process [11]. Under normal circumstances autophagy clears damaged mitochondria in order to abolish cell death. Therefore two autophagic assays were tested: the effect of 3-methyladenine (3-MA) and the expression of LC3. 3-MA is an autophagy inhibitor which mediates class III of the PI3K essential for formation of autophagosomes [12]. As a positive control for autophagy induction, HEK293T cells were starved in PBS pretreated either with or without 10 mM 3-MA for 3-4 hours. Initiation of autophagy was confirmed by light microscopy. This clearly demonstrated the induction of autophagy as shown by the cell morphology (data not shown). Samples were collected to measure the cell viability by flow cytometry. Addition of 10 mM 3-MA did not increase the viability in mut*DFNA5*-transfected HEK293T cells as compared to untransfected HEK293T cells (Fig. 7a).

LC3 is a marker for autophagic flux involved in the formation of the autophagosomes. Upon induction of autophagy, LC3-I is converted to LC3-II indicating autophagosome formation. Total protein lysates of transfected HEK293T cells were collected and the LC3 conversion was investigated by western blot. LC3-I however was converted to LC3-II (Fig. 7b) but no significant differences were seen upon comparison of the control, wtDFNA5 or mutDFNA5 protein samples (Fig. 7b). Due to lack of proper autophagic flux inhibitors like bafilomycin and to the dual specificity and non-autophagy-related role of 3-MA, a correct interpretation of the LC3-II protein is however difficult [12-14].

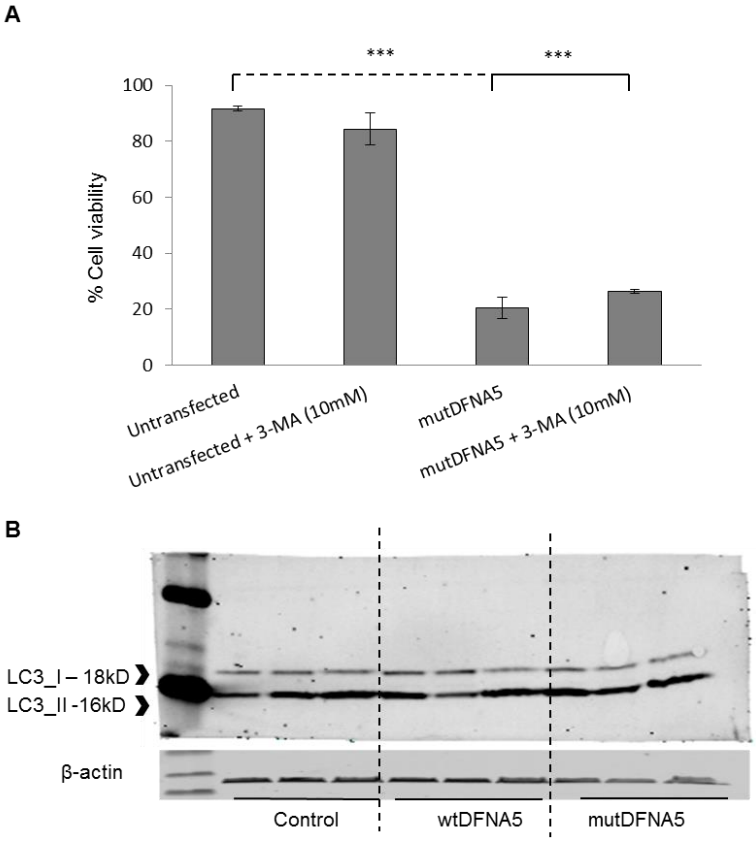

Fig. 7: Autophagic assays. a. 10 µM 3-MA was added overnight for 16 h to mut*DFNA5*-transfected and untransfected HEK293T cells. Pretreated HEK293T cells were incubated for 3-4 hours with PBS as a positive control for autophagy induction (data not shown). Cell viability was measured by flow cytometry using PI as a cell death marker. **b.** Western Blot analysis of the LC3 protein, a marker for autophagic flux, in HEK293T transfected cell lines. Conversion of LC3-I to LC3-II was seen in all samples, suggesting no differences in autophagy level between mutDFNA5, control and wtDFNA5. * denotes a significant difference in cell viability in mut*DFNA5* compared to untransfected HEK293T cells. ***: p<0.01; 3-MA, 3-methyladenine.

4.5 Discussion

This study investigated the role of DFNA5 in programmed cell death in HEK293T cells. As previous research in yeast indicated a link between DFNA5 and several mitochondrial proteins (see chapter II), we examined the role of the mitochondria in human cell lines in more detail. MutDFNA5-mediated cell death was indeed related to mitochondrial dysfunction as increased ROS production and partial loss of the mitochondrial membrane potential (ΔΨ) were observed. Decreased ΔΨ could indicate mitochondrial permeabilisation suggesting a mitochondrial role in mutDFNA5-induced cell death. Mitochondrial permeabilisation is a key process of the intrinsic apoptotic pathway and although enhanced cytosolic cytochrome c was demonstrated upon mut*DFNA5* transfection, caspase activation seems to be absent as demonstrated by the lack of caspase-3 cleavage. Furthermore, release of the matrix citrate synthase, a marker for intact mitochondria [15], and the accumulation of damaged mitochondria, visualised by fluorescence microscopy, suggested the presence of damaged mitochondria. As the cytosolic release of cytochrome c and citrate synthase is limited and both are still present in the mitochondria, mitochondrial disintegration seems to be partial and does not represent an end-stage in this cell death process.

Due to the lack of caspase cleavage and the increased release of cytochrome c and citrate synthase, it can be concluded that the DFNA5-induced cell death is not mediated by mitochondrial permeabilisation, but the presence of damaged mitochondria will most likely result in the release of those factors. How mutDFNA5 is related to this accumulation of damaged mitochondria resulting in mitochondrial collapse remains unknown at this moment.

In addition, the absence of the DNA-laddering pattern suggests the lack of specific DNA fragmentation, contrasting the former TUNEL staining [5]. TUNEL however, is a very unspecific method to measure DNA fragmentation. A smear of DNA, which was slightly visible in mutDFNA5 DNA samples, can also give positive TUNEL results. The previous positive TUNEL staining experiments might be the result of unspecific, random DNA cleavage by nucleases released during the cell death process [5]. Moreover, upon re-evaluation of the AnnexinV/PI results, the increase in AnnexinV

positive cells was only seen in double stained, AnnexinV/PI cells. Single AnnexinV positive cells however were not increased, but only a small increase in single PI-stained cells could be observed (data not shown). Double stained AnnexinV/PI cells and a small increase in single PI-stained cells rather indicate a non-apoptotic form of cell death induced by mutDFNA5. Based on these results, we concluded that mutDFNA5 does not induce cell death through the caspases-dependent apoptotic pathway. Due to these conclusions, we performed some basic cell death assays to distinguish between different types of non-apoptotic cell death, namely ferroptosis and necroptosis, both independent on caspases and associated with enhanced ROS [7, 10, 16, 17].

Ferroptosis is morphological and biochemical distinct from other forms of PCD, such as apoptosis, necrosis or autophagy. Ferroptosis is characterised by smaller mitochondria and iron-dependent accumulation of lethal lipid ROS. Exploration of the genetic basis of ferroptosis by a custom arrayed shRNA library targeting mitochondrial genes identified a role for citrate synthase [7]. Citrate synthase is a mitochondrial matrix protein involved in mitochondrial fatty acid metabolism [15]. Silencing of citrate synthase specifically abolished erastin-induced ferroptosis, suggesting a role for citrate synthase and mitochondrial lipid synthesis in ferroptosis [7].
Ferroptosis is induced by the depletion of glutathione peroxidase 4, due to impaired cystine uptake, abolishing the cellular anti-oxidant response [18]. Due to increased ROS production, the mitochondrial collapse and the release of citrate synthase, we wondered whether ferroptosis could be involved in mutDFNA5-induced cell death. However, addition of ferrostatin, a ferroptosis inhibitor [7, 10], did not ameliorate cell viability of mut*DFNA5*-transfected HEK293T cells. The ferrostatin inducer erastin however, did not decrease cell viability of untransfected HEK293T cells. This could be due to the fact that HEK293T cell lines do not contain a mutation in the RAS oncogene. Erastin was initially identified as a component selectively lethal to RAS-mutant cell lines which could explain these results [7]. Although we are currently investigating a RAS mutant tumour cell line determining the

efficacy of erastin, the lack of increased viability of mut*DFNA5*-transfected HEK293T cells, makes a role for ferroptosis highly uncertain.

Necroptosis is a non-apoptotic caspase-independent form of cell death initiated by death receptors ligands and mediated by the kinase activity of RIPK1. RIPK1 can also induce apoptosis by recruiting caspase-8. However, in the absence of caspase activity, RIPK1 can mediate necroptosis through its kinase domain. Activated RIPK1 forms a complex with MLKL and RIPK3, a kinase essential for necroptosis [16, 19, 20]. Necroptosis is characterised by enhanced ROS production, no caspase-3 cleavage and TUNEL positivity due to plasma membrane rupture [21]. Therefore, we further investigated the role of necroptosis by the use of Nec-1s, a specific RIPK1 kinase inhibitor able to inhibit necroptosis. Addition of Nec-1s did not have a significant effect on cell viability of mut*DFNA5*-transfected HEK293T cells. Because Nec-1s was shown to be able to block necroptosis by inhibiting the kinase activity of RIPK1, it is believed that RIPK1 is required to activate RIPK3, an essential executioner of necroptosis [19, 22, 23] . However, due to the lack of RIPK3 and MLKL in HEK293T, mutDFNA5 is probably not able to induce necroptosis in HEK293T cells, explaining the lack of an effect of the inhibition of RIPK1 by Nec-1s. Therefore, a role of necroptosis in mutDFNA5-induced cell death can most likely be excluded.

Next to apoptosis, different forms of PCD can be mediated through the mitochondria. Mitochondria have different protein quality control mechanisms and one of them is mitophagy, a selective form of autophagy. Mitophagy is a clearance system mediating degradation of the complete mitochondria protein content. Functional mitochondrial impairment and permeabilisation can promote mitophagy acting as a quality control process. Several neurodegenerative diseases and lysosomal storage disorders have been correlated with defective quality control mechanisms and dysfunctional mitochondria. These mitochondria are characterised by loss of the $\Delta\Psi$ and increased ROS production [24]. Moreover, mitochondrial dynamics and enhanced mitochondrial fission mediating isolation of damaged mitochondria are closely related to mitophagy [25, 26]. Although

we investigated the role of LC3 in mutDFNA5-induced cell death, due to the lack of proper autophagic inhibitors and the unequal sensitivity of the antibody for LC3-II, correct interpretation of the LC3-I/ LC3-II ratio is currently difficult. Therefore it could be interesting to repeat the experiments with the addition of either bafilomycin A or chloroquine, two frequently used autophagic flux inhibitors, to more specifically determine the amount of LC3-II and hence the role of autophagy. Additionally, a co-staining of autophagosomes by LC3-GFP and the mitochondria using a specific mitotracker can provide extra information on the role of mitophagy. Degradation of the mitochondrial network and accumulation of damaged mitochondria described in this study, can indeed point to a correlation with mitophagy. Proper turnover of the mitochondria is essential for energy homeostasis and deregulation of mitophagy results in accumulation of damaged mitochondria. This will ultimately lead to (neuronal) cell death as demonstrated in several neurodegenerative diseases [27]. Finally, electron microscopy (EM) can also be used as a quantitative identification method for autophagic vesicles and the morphology of the mitochondria in more particular [28]. Although this has not yet been performed, this is something which certainly has to be explored in the future.

In addition to mitophagy, mutDFNA5-associated cell death can also be mediated by mitoptosis, programmed destruction of the mitochondria which differs from mitophagy. Mitoptosis is characterised by extensive mitochondrial fragmentation, does not require extra mitochondrial proteins and is caspase independent [29, 30]. Although mitoptosis can stimulate mitophagy, mitoptosis can occur in a non-autophagic mode of cell death by the formation of mitoptosomes which are secreted into the extracellular space [31, 32]. Due to the presence of fragmented mitochondria and the lack of caspase cleavage, the presence of mitoptosis is a reasonable possibility which has to be further investigated in the future.

In conclusion, DFNA5-induced cell death is characterised by accumulation of damaged mitochondria as demonstrated by disintegration of the mitochondrial network and the release of several mitochondrial proteins.

Despite the observations of dysfunctional mitochondria, their relation to mutDFNA5 needs to be further investigated.

4.6 References

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Part III

General discussion and future perspectives

5 General discussion and future perspectives

DFNA5 was originally identified as a gene responsible for an autosomal dominant non-syndromic form of hearing loss (HL) [1]. Nowadays, eight families have been identified with mutations in *DFNA5* related to HL. Four of these mutations differ at the genomic level, but they all result in the skipping of exon 8 leading to an immature truncated protein [1-4]. Besides HL, *DFNA5* has been associated with several different forms of cancer, such as breast, colorectal, gastric and melanoma cancer. Here, *DFNA5* is epigenetically inactivated by hypermethylation, resulting in enhanced tumour growth. Based on this, *DFNA5* has been identified as a potential tumour suppressor gene [5-7].

At the beginning of my PhD thesis, the knowledge of the function of DFNA5 was limited. Previous studies on DFNA5 revealed that transfection of an expression construct for the DFNA5 mutation induced a growth defect in mammalian cell lines and in fission yeast [8, 9]. The domain responsible for cell death induction was assigned to the first globular part of DFNA5, a part present both in wt*DFNA5* and in mut*DFNA5* [9]. Apart from small homology domains with *Pejvakin* and the yeast *Mcm10* gene, *DFNA5* did not have a high degree of homology with other genes, making functional assumptions not evident. As this identified growth defect seemed to be at least partially conserved among eukaryotes as shown by Gregan *et al.* [8], further research on DFNA5-related cell death mechanisms was started in a yeast model, namely *Saccharomyces cerevisiae*. In a second stage, HEK293T cell lines were used as a model.

5.1 Yeast as a humanised model system for DFNA5

This project initially mainly focused on apoptosis, as a link to apoptosis was previously demonstrated in HEK293T cells [9]. Apoptosis is a complex mechanism highly connected with other forms of PCD. A simplified version of apoptosis exists in yeast, making the interpretation of results slightly less complicated. However, this needs to be taken with caution as biased interpretation of data might occur. The first aim of this thesis was to generate and evaluate the new *DFNA5* yeast model. Transformation of

mut*DFNA5* in yeast indeed induced a growth defect associated with PCD and this toxicity was still confined to the first globular domain as described in chapter II. A mixed form of PCD was however present, as both apoptotic and necrotic yeast cells accumulated upon mut*DFNA5* expression. At first this seemed to be in contrast with the human cell line results. The initial association of apoptosis with DFNA5 however, turned out to be premature as later results pointed to a necrotic form of cell death. The absence of the DNA laddering pattern and the increase in double AnnexinV/PI and single PI-stained cells supports this hypothesis. Furthermore, yeast created the basis for future experiments in HEK293T supporting the value of the DFNA5 yeast model. Several mitochondria-related proteins associated with DFNA5-induced cell death were characterised in yeast. Proteins like Fis1, Por1, Aac1 and Aac3 were identified to mediate the DFNA5-related growth defect. All these proteins are related to the mitochondrial dynamics and the permeabilisation process. Moreover, in yeast we observed a partial co-localisation of mutDFNA5 and the mitochondria, and the loss of an intact mitochondrial network. The importance of the mitochondria was indeed later confirmed in HEK293T cells by the loss of an intact mitochondrial network and the release of certain mitochondrial proteins (described in chapter IV).

The region responsible for the apoptotic effect was assigned to the first globular domain, as previously identified in HEK293T cells [9]. Interestingly, the PCD-inducing capacity of this region was also present in yeast cells. Substitution of the second, highly conserved, hydrophobic amino acid of this domain abolished the cell death-inducing capacity of this N-terminal globular domain both in yeast and in HEK293T cells. This confirms the relevance of the yeast model.

Based on the conserved role of the mitochondria and the PCD-inducing capacity of the first globular domain in mutDFNA5-induced cell death, yeast seemed to be a suitable model to investigate DFNA5-related mechanisms. However, as this model also has some disadvantages (one major limitation is

the lack of the extrinsic apoptotic pathway), these results needed to be further validated in human cell lines.

5.2 MutDFNA5 induces mitochondrial cell death mediated by MAPK

The second part of this thesis was based on the previous yeast results and was performed in human cell lines. This part investigated several aspects of the mitochondria. Several mitochondrial assays demonstrated altered mitochondrial morphology and function induced by mutDFNA5. This was shown by fluorescence microscopy and the cytosolic release of both cytochrome c and citrate synthase. Furthermore, a transcriptomic analysis revealed MAPK activation induced by mut*DFNA5*. Although mitochondrial alteration mediated by MAPK pathways is well known, numerous mechanisms are still possible. The most common interference is through the BCL2 protein family. Both anti- and pro-apoptotic proteins can respectively be inhibited and activated by the JNK proteins. This interference influences the critical equilibrium of the BCL2 family tipping the balance to pro-apoptosis [10, 11].

To investigate the link between the mitochondria and the MAPK pathways, it would be interesting to investigate whether either JNK or ERK are localised in the mitochondria as mitochondrial translocation of both proteins was observed upon cell death induction by other researchers [12-16]. This can be either direct, as shown for ERK, which was found associated with mitochondrial proteins [17, 18]. It can also be indirect by interaction with the OMM, which was demonstrated for JNK [19]. This mitochondrial localisation has been associated with the release of several apoptotic factors, such as Smac and cytochrome c, and addition of specific JNK inhibitors reduced the stress-induced release of Smac and cytochrome c. ERK and JNK have also been correlated with oxidative stress and mitochondrial dysfunction, two features observed upon mutDFNA5-induced cell death. In previous studies, oxidative stress enhanced the phosphorylation of either ERK or JNK, followed by reduced ATP production and lower respiration [14, 20]. All these findings provide a direct link

between MAPK activation and the mitochondrial death machinery. ATP depletion also shows that the MAPK pathway can interfere with the mitochondrial metabolism, regulating mitochondrial bio-energetics. Altered cellular and mitochondrial energetics has a direct effect on mitochondrial morphology as shown by diverse studies [21-23]. Alterations in metabolic flux, such as elevated glucose levels, free fatty acids or Ca^{2+} supply, have been shown to have a direct effect on ROS production and subsequently on mitochondrial morphology. Mitochondrial fragmentation and ROS production are hence tightly associated with mitochondrial bio-energetics which need to be closely regulated [21, 22, 24, 25]. The determination of the ATP level in mut*DFNA5*-transfected cell lines could therefore give an idea about the bio-energetics of the mitochondria. As described in chapter II, research in yeast identified two proteins involved in ATP transport, namely Aac1 and Aac3. The Aac proteins function as ADP/ATP transporters and mediate ATP release to the cytosol under healthy cellular conditions. This finding could point to altered ATP levels in mut*DFNA5*-transformed yeast cells.

5.3 The role of other forms of PCD

This project initially mainly focused on the investigation of apoptosis as previous research pointed in this direction [9]. However, many different forms of PCD co-exist and it is often not evident to pinpoint one particular form of PCD. Therefore, we reinvestigated the role of apoptosis, as well as other forms of PCD.

Apoptosis is a cellular mechanism that can lead to death of stressed cells and can indicate an end-stage in the struggle for life, preceded by other processes. Autophagy on the other hand, is often regarded as a pro-survival mechanism, trying to prevent cell death by recycling and specifically eliminating damaged or superfluous organelles. It elicits an adaptive stress response avoiding cell death, or it can result in autophagic cell death indicating a link to other forms of PCD. This crosstalk however, is very complex and both apoptosis and necrosis, can be preceded, inhibited or activated, by autophagy [26, 27].

We did not investigate in detail the role of autophagy and preliminary experiments studying the role of LC3 were inconclusive. Therefore, it is worthwhile to investigate the role of autophagy in more detail, especially at earlier time points where autophagy could precede cell death. As autophagy is a conserved mechanism among eukaryotes [28, 29], studying the role of several *Atg* genes in *DFNA5*-transformed yeast cells, could certainly uncover interesting findings about the importance of this cellular process.

Much is still unknown about the crosstalk between PCD and autophagy, but several proteins have been identified to play a dual role in for example PCD and autophagy. The death-associated protein kinase (DAPK) for example, was first identified as a positive mediator of PCD, and later as an activator of autophagy [30-32]. DAPK becomes activated by oxidative stress. Activated DAPK regulates JNK signalling and is required for caspase-independent necrotic cell death [33]. For autophagy, DAPK has been associated with the phosphorylation of Vps34, a kinase indispensable for autophagosome formation [34].

This is just one of the many examples which demonstrate the complex crosstalk between autophagy and PCD. They both can be induced by common stimuli resulting in either a mixed phenotype, or excluding each other by mutual inhibition. This complex relationship clearly indicates the importance of investigating the role of autophagy in mechanisms related to PCD, such as DFNA5.

Additionally, increased ROS production and loss of the mitochondrial network can also suggest the presence of mitoptosis or programmed destruction of the mitochondria, a mitochondrial suicide mechanism [35, 36]. This can be mediated by DRP-1, a mitochondrial fission protein inducing mitochondrial fragmentation, or through protease inhibitors [37, 38]. During mitoptosis, mitochondria appear condensed, followed by swelling, rupture of the OMM and mitochondrial fragmentation. Clustering of fragmented mitochondria and membrane sequestration will form mitoptotic bodies. Inside this mitoptotic body, fragmented mitochondria are further degraded and finally released in the extracellular space leading to a non-autophagic-related mitoptotic cell death [35, 37, 39]. Programmed destruction of the

mitochondria can also be followed by activation of selective mitochondrial autophagy, known as mitophagy [35, 40]. It has been shown previously that mitophagy is induced by dissipation of the Δψ and mediated by JNK, linking autophagy and MAPK-signalling pathways [38].

5.4 Role of the ER in mutDFNA5-induced cell death?

This study revealed a role for the mitochondria and suggested a role for the ER in mutDFNA5-induced cell death mechanisms in both model organisms. It would therefore be interesting to investigate the connection between those two organelles in mutDFNA5-associated cell death and to determine whether the GO-identified processes (chapter III), like protein folding, are related to ER stress.

These two organelles are highly interconnected by mitochondria-associated membrane channels, mediating mitochondrial supply of metabolites and lipids. ER stress has therefore a detrimental effect on mitochondrial metabolism and hence the cellular energy status (reviewed in [41, 42]). ER stress can induce cell death through the mitochondria and is characterised by accumulation of unfolded proteins in the lumen. Unfolded proteins are sensed by three different transmembrane proteins, triggering an adaptive response [43]. One of the downstream events is the up-regulation of CHOP and cleavage of XBP-1, two markers for ER stress. Additionally, an increased amount of polyubiquitinated proteins, Ca^{2+} release through the IP3-ER Ca^{2+} channel and eIF2α phosphorylation, are also typical hallmarks of ER stress [44, 45].

Interestingly, CHOP can be directly induced by activated JNK, implying a link between the MAPK pathway and ER stress [46, 47]. JNK is activated by Ask1, a kinase involved in the receptor-mediated branch of apoptosis. Upon ER stress, Ask1 is recruited by one of the three ER transmembrane protein sensors [48]. In addition to CHOP activation, activation of the IRE1α branch is also involved in JNK signalling [48, 49]. IRE1α activation by ER stress leads to the recruitment of the TRAF adaptor protein leading to JNK activation [50, 51]. The IRE1α pathway is the only conserved ER stress pathway in yeast and can induce cleavage of the XBP-1 (Hac1 in yeast) mRNA [52]. As an association with GO pathways related to protein folding and the ER were

seen in both model organisms, the investigation of the role of the IRE1α pathway could be a good starting point for future research.

Release of Ca^{2+} from the ER by the IP3-receptor can directly induce mitochondrial permeabilisation and the IP3-receptor can be modulated by several BCL2 family proteins, providing a link between the mitochondria and the ER [53, 54]. Interestingly, cytochrome c released from the mitochondria has been shown to bind to the IP3-channel. This can regulate the Ca^{2+} release and amplify the mitochondria-related cell death signal [55]. Sustained ER stress can indeed modulate mitochondrial morphology. The release of Ca^{2+} seems to play a prominent role in this process and this is often accompanied by mitochondrial fragmentation [41, 56, 57]. However, we did not measure the Ca^{2+} levels in this study. Furthermore, ER stress has also been linked to increased oxidative stress which further supports a putative role of the ER in mutDFNA5-associated cell death [58].

These data demonstrate a link between the MAPK pathways and the induction of mitochondria-related cell death. The investigation of certain ER markers, such as CHOP, XBP-1 cleavage or the Ca^{2+} release by the ER, would reveal extra information on the role of the ER in mutDFNA5-induced cell death.

But if ER stress would be present, how would it relate to mutDFNA5? The ER is responsible for protein folding and protein quality control of roughly one-quarter of the proteome (proteins of the secretory pathway) [59]. Misfolded proteins, leading to ER stress, can either elicit an unfolded protein response in order to increase the folding capacity of the ER. Alternatively they can initiate an ER-associated degradation (ERAD) pathway, recognizing terminally misfolded proteins. Both responses are coordinated and mediate each other [60-63]. Different ERAD systems exist both in yeast and human cell lines, namely ERAD-M, ERAD-L and ERAD-C responsible for degradation of respectively misfolded transmembrane, luminal or cytosolic parts of transmembrane proteins of the ER [64, 65]. The presence of a granulated expression pattern of mutDFNA5 in HEK293T and in yeast cells, in contrast

to an evenly distributed cytoplasmic expression of wtDFNA5, could suggest impaired protein folding leading to aggregate formation. Due to the association of GO terms-related to the ER and protein folding, this could suggest a failure of the protein quality control in the ER. However, as far as we known, DFNA5 is a cytoplasmic protein, not located in the ER or any other secretory organelle.

How aggregated mutDFNA5 would mediate ERAD is uncertain at this moment, but examples of the requirement of ERAD-C for the degradation of non-ER proteins do exist in yeast [66-69]. The role of ERAD-C in yeast depends on the presence of a specific degradation sequence in the substrate recognised by Doa1, the yeast homologue of TEB4 (MARCH6). Doa1 is an E3 ligase mediating the ubiquitination process essential for degradation of the misfolded substrates [66, 68]. This degradation is dependent on Ubc6 and Ubc7, two ubiquitin-conjugating enzymes [67].

There is one example of a link between ERAD and cytosolic degradation of a non-ER protein in human cell lines [70]. This study demonstrated proteasomal degradation of aberrantly located p53 mediated by Synoviolin, an ER-resident E3 ubiquitin ligase, under mild ER stress. It remains to be investigated whether other ERAD-related components are involved in this degradation. This could indicate whether this degradation is indeed related to ERAD or if this is due to an additional non-ERAD-related feature of Synoviolin.

5.5 Role of ER stress in the pathology of hearing loss

Despite the limited knowledge about the correlation of mutDFNA5 with ER stress, certain forms of HL have already been correlated with the presence of ER stress. The Usher syndrome for example, a hereditary form of hearing loss combined with visual impairment, is associated with enhanced ER stress that promotes apoptosis. Different Usher-associated proteins of the secretory pathway preassemble on the ER before trafficking to Golgi. This cooperation is essential to shape the sensory hair cells and stereocilia. Mutations in one of these genes lead to protein trafficking defects and mislocalisation of several Usher-associated proteins, proteins related to

either the Usher type 1 or 2 clinical phenotype. This mislocalisation induces ER stress followed by apoptosis of the hair cells [71].

In addition to the hereditary forms of HL, ototoxicity (HL due to the use of pharmaceuticals such as aminoglycoside antibiotics and platinum-based chemotherapeutics) was also shown to be correlated with ER stress-dependent pathways. Certain pain relievers, contributing to tinnitus and progressive bilateral sensorineural HL, were shown to induce ROS overproduction, altered ER morphology and changes in ER stress markers, such as CHOP [72]. The role of the ER however, remains to be elucidated in DFNA5-related cell death mechanisms.

5.6 References

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Appendix

IV. Curriculum vitae

General information

Name:	Sofie Van Rossom
Date of birth:	21 March 1986, Ekeren (Antwerp)
Nationality:	Belgian
Home address:	Van Trierstraat 6 2018 Antwerpen
Work address:	University of Antwerp Department of Medical Genetics Prins Boudewijnlaan 43 2650 Edegem sofie.vanrossom@uantwerpen.be

Education

2009-recent	PhD student Biochemistry and biotechnology Department of Medical Genetics University of Antwerp Supervisor: Prof. Guy Van Camp Faculty of Biology K U Leuven Supervisor: Prof. Joris Winderickx
2009	Certificate to conduct experiments with laboratory animals (Felasa C), University of Antwerp
2007-2009	Master in Biohemistry and Biotechnology University of Antwerp Finality: research molecular and cellular gene bio-technology
2004-2007	Bachelor in Biochemistry and Biotechnology University of Antwerp

Grants

2010-2014 PhD fellowship from the Instituut voor Wetenschap en Technologie (IWT)

Publications

Van Rossom S, Op de Beeck K, Franssens V, Swinnen E, Schepers A, Ghillebert R, Caldara M, Van Camp G and Winderickx J. Frontiers in oncology (2012) Jul 25(2) 77. The splicing mutant of the human tumour suppressor protein DFNA5 induces programmed cell death when expressed in the yeast *Saccharomyces cerevisiae. Front Oncol, 2012. **2**: p. 77.*

Van Rossom S, Op de Beeck K, Hristovska V, Franssens V, Swinnen E, Schepers A, Ghillebert R, Caldara M, Van Camp G and Winderickx J. Mutant DFNA5 induces ER stress by mitochondrial and MAPK-related pathways. *Submitted*

Van Rossom S, Goossens V, Op de Beeck K, Martinet W, Vandenabeele P, Winderickx J and Van Camp G. Mutant DFNA5-induced cell death is mediated dysfunctional mitochondria**.**
Work in progress

Oral presentations

Van Rossom S, Op de Beeck K, Franssens V, Swinnen E, Schepers A, Ghillebert R, Caldara M, Van Camp G and Winderickx J. The splicing mutant of the human tumor suppressor protein DFNA5 induces programmed cell death when expressed in the yeast *Saccharomyces cerevisiae*. 9th International Meeting on Yeast Apoptosis, Rome, Italy, September 2012

Van Rossom S, Op de Beeck K, Franssens V, Winderickx J and Van Camp G. The apoptosis inducing gene DFNA5 in deafness and cancer: a delicate balance between too much and too little. FBD Researchday, Wilrijk, Belgium, Octobre 2013.

Poster presentations

Van Rossom S, Op de Beeck K, Franssens V, Winderickx J and Van Camp G. The elucidation of the apoptotic mechanisms of DFNA5 based on humanised yeast models. Yeasterday, K.U.Leuven, May 2010

Van Rossom S, Op de Beeck K, Franssens V, Winderickx J and Van Camp G. First steps towards the elucidation of the apoptotic mechanisms of DFNA5 based on humanised yeast models. 8th International Meeting on Yeast Apoptosis, Kent, UK, May 2011

Van Rossom S, Op de Beeck K, Franssens V, Winderickx J and Van Camp G. Elucidation of the apoptotic mechanisms of DFNA5 using humanised yeast models. 12th Annual meeting of the Belgian Society of Human Genetics, Luik, Belgium, March 2012.

Van Rossom S, Op de Beeck K, Franssens V, Winderickx J and Van Camp G. The elucidation of the pathophysiology of DFNA5 in a humanised yeast model. 13th Annual meeting of the Belgian Society of Human Genetics, Brussel, Belgium, March 2013.

Van Rossom S, Op de Beeck K, Franssens V, Winderickx J and Van Camp G. DFNA5 induces apoptosis in mammalian cells through a mitochondrial pathway
14th Annual meeting of the Belgian Society of Human Genetics, Antwerp, Belgium, February 2014.

Scientific courses

EUROHEAR Theoretical training: on therapy. Padova, Italy, September 2009

ICYSB2011, International Course in Yeast System Biology organised by Federation of the European Biochemical Societies, Gothenburg, Sweden, 6-23 June 2011

BioSCenter Doctoral School Course Life Sciences Technology Watch, Leuven-Heverlee, Belgium, September 2011-January 2012

V. Supplementary Material

V.1 Supplementary Material Chapter II

Table 2.1: Strain list

Strain	Genotype	Reference/Source
BY 4741	Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	*[20]*
aac1Δ	BY4741*YMR056c*::kanMX	EUROSCARF
aac3Δ	BY4741*YBR085w*::kanMX4	EUROSCARF
aif1Δ	BY4741*YNR074c*::kanMX4	EUROSCARF
dnm1Δ	BY4741*YLL001w*::kanMX4	EUROSCARF
fis1Δ	BY4741*YIL065c*::kanMX4	EUROSCARF
mca1Δ	BY4741*YOR197w*::kanMX4	EUROSCARF
mdv1Δ	BY4741*YJL112w*::kanMX4	EUROSCARF
nma111Δ	BY4741*YNL123w*::kanMX4	EUROSCARF
nuc1Δ	BY4741*YJL208c*::kanMX4	EUROSCARF
por1Δ	BY4741*YNL055c*::kanMX4	EUROSCARF
por2Δ	BY4741*YIL114c*::kanMX4	EUROSCARF
rny1Δ	BY4741*YPL123c*::kanMX4	EUROSCARF
snl1Δ	BY4741*YIL016w*::kanMX4	EUROSCARF
tdh2Δ	BY4741*YJR009c*::kanMX4	EUROSCARF
tdh3Δ	BY4741*YGR192c*::kanMX4	EUROSCARF
tim18Δ	BY4741*YOR297c*::kanMX4	EUROSCARF
ymr074cΔ	BY4741*YMR074c*::kanMX4	EUROSCARF
ysp1Δ	BY4741*YHR155w*::kanMX4	EUROSCARF

Table 2.2: Primer pairs

Construct	Forward primer	Reverse primer
wtDFNA5-HA	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	GACCGGTGGATCCCGTGAATGTTCTCTGCCTAAAGC
mutDFNA5-HA	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	GACCGGTGGATCCCGAGGTTGGGTCTTCAAGATCAG
wtDFNA5-EGFP	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	TATATATAGTCGACGCTTGTACAGCTCGTCCATGCC
mutDFNA5-EGFP	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	TATATATAGTCGACGCTTGTACAGCTCGTCCATGCC
DFNA5 domain A	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	CGGTGGATCCCGGTCCAGGTAGACAGAGTCAAT
DFNA5 domain B	AAGCTTCGAATTCTGGCCACCATGGACCCCCTGGTCTTTCGAGAG	GACCGGTGGATCCCGTGAATGTTCTCTGCCTAAAGC
HCA-F2R mutant*	GAGCTCAAGCTTCGAATTCTGATGCGTGCCAAAGCAACCAGG	CCTGGTTGCTTTGGCACGCATCAGAATTCGAAGCTTGAGCTCG
HCA-A3R mutant*	CGAGCTCAAGCTTCGAATTCTGATGTTTCGTAAAGCAACCAGG	CCTGGTTGCTTTACGAAACATCAGAATTCGAAGCTTGAGCTCG

Restriction sites used for cloning are underlined
*=site-directed mutagenesis primers

Table 2.3: Quantification of growth*

Strain	Function	T½ Control	T½ wtDFNA5	T½ mutDFNA5	ΔT½ wtDFNA5-Control	ΔT½ mutDFNA5-Control	%ΔT½ mutDFNA5-wtDFNA5
BY4741 (HA-tagged)		19.83 ± 0.52	22.83 ± 0.26	31.38 ± 0.48	3.00 ± 0.24	11.54 ± 0.32	100.00 ± 1.42
BY4741 (EGFP-tagged)		20.30 ± 1.15	24.90 ± 2.61	35.88 ± 3.54	4.60 ± 1.27	15.58 ± 1.84	128.49 ± 8.60
aac1Δ	Mitochondrial ADP/ATP Carrier	31.30 ± 1.20	28.13 ± 5.36	31.08 ± 1.36	-3.18 ± 2.73	-0.22 ± 0.77	34.63 ± 11.17
aac3Δ	Mitochondrial ADP/ATP Carrier	24.70 ± 0.45	27.00 ± 0.82	29.40 ± 1.67	2.30 ± 0.45	4.70 ± 0.77	28.10 ± 3.37
aif1Δ	Mitochondrial Apoptosis inducing factor	19.60 ± 0.22	23.13 ± 0.25	33.00 ± 0.82	3.53 ± 0.16	13.40 ± 0.42	115.61 ± 1.76
dnm1Δ	Dynamin-related GTPase, mitochondrial fission	20.20 ± 0.27	22.33 ± 1.61	31.50 ± 2.38	2.13 ± 0.94	11.30 ± 1.20	107.32 ± 6.07
fis1Δ	Mitochondrial membrane fission	26.70 ± 1.10	34.38 ± 1.80	48.67 ± 1.15	7.68 ± 1.02	21.97 ± 0.83	167.32 ± 5.26
mca1Δ	Putative cysteine protease, metacaspase	21.80 ± 0.27	24.30 ± 0.45	34.50 ± 2.27	2.50 ± 0.23	12.70 ± 1.14	119.41 ± 4.54
mdv1Δ	Dmn1 adaptor protein, mitochondrial fission	20.40 ± 1.34	24.38 ± 0.25	31.90 ± 2.01	3.98 ± 0.61	11.50 ± 1.08	88.10 ± 4.66
nma111Δ	Omi1/HtrA2 Orthologue, Serine protease	20.00 ± 0.00	23.67 ± 0.58	31.25 ± 2.22	3.67 ± 0.33	11.25 ± 1.11	88.78 ± 4.82
nuc1Δ	Mitochondrial nuclease, Endo G orthologue	22.38 ± 2.14	24.10 ± 0.55	33.67 ± 0.58	1.73 ± 1.10	11.29 ± 1.12	112.00 ± 6.54
por1Δ	Mitochondrial porin, VDAC homologue	20.50 ± 1.50	25.40 ± 0.65	40.00 ± 1.38	4.90 ± 0.91	19.50 ± 1.03	170.93 ± 5.53

Table 2.3 continued: Quantification of growth*

Strain	Function	T½ Control	T½ wtDFNA5	T½ mutDFNA5	ΔT½ wtDFNA5-Control	ΔT½ mutDFNA5-Control	%ΔT½ mutDFNA5-wtDFNA5
por2Δ	Putative mitochondrial porin, VDAC homologue	21.20 ± 0.45	23.90 ± 0.42	31.30 ± 2.49	2.70 ± 0.27	10.10 ± 1.13	86.63 ± 4.31
rny1Δ	Ribonuclease from yeast	20.90 ± 0.55	22.80 ± 0.57	33.90 ± 2.13	1.90 ± 0.35	13.00 ± 0.98	129.95 ± 3.87
snl1Δ	Suppressor of nup116-C lethal, Bag-1 homologue	18.60 ± 0.89	20.38 ± 0.25	28.00 ± 0.00	1.78 ± 0.42	9.40 ± 0.40	89.27 ± 2.33
tdh2Δ	Triose-phosphate dehydrogenase	20.13 ± 0.25	23.00 ± 0.50	31.00 ± 1.54	2.88 ± 0.31	10.88 ± 0.70	93.66 ± 3.07
tdh3Δ	Triose-phosphate dehydrogenase	22.00 ± 0.00	23.88 ± 0.25	35.50 ± 1.00	1.88 ± 0.13	13.50 ± 0.50	136.10 ± 2.13
tim18Δ	Translocase of the inner mitochondrial membrane	21.40 ± 0.42	25.50 ± 0.50	33.50 ± 0;58	4.10 ± 0.34	12.10 ± 0.34	93.66 ± 1.96
ymr074cΔ	Protein with homology to human PDCD5	20.75 ± 0.50	23.70 ± 0.57	29.00 ± 3.32	2.95 ± 0.36	8.25 ± 1.50	62.05 ± 6.03
yspΔ	Yeast suicide protein	20.00 ± 0.00	23.40 ± 1.39	34.10 ± 1.95	3.40 ± 0.62	14.10 ± 0.87	125.27 ± 4.18

T½: time to reach half maximal optical density (hours)

ΔT½: difference in time to reach half maximal optical density (hours)

*Values are expressed as mean ± standard deviation.

V.2 Supplementary Material Chapter III

Table 3.1: Primer sequences for RT-PCR

Gene	Fw_sequence	Rv_sequence
TM7SF2	TCT ACA TGA AGG CGC AGG TAG C	AGT CGT AAA TCG GAT TGC CTG AG
UCP2	TCT CCC AAT GTT GCT CGT AAT GCC	AAG TGG CAA GGG AGG TCA TCT GTC
VPS33B	TGT GGA TCT GCT GAG CAT GGA AC	TCC AAC GCT GAT CTC CTT CCA G
FOSB	GAG AGC TGG TAG TTA GTA GCA TGT GA	AAT TCC AAT AAT GAA CCC AAT AGA TTA
EGR1	GCA CCT GAC CGC AGA GTC TT	AGT GGT TTG GCT GGG GTA ACT
HMBS_HHG	GGC AAT GCG GCT GCA A	GGG TAC CCA CGC GAA TCA C
GAPDH_HHG	TGC ACC ACC AAC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG
RPL13A_HHG	CCT GGA GTT TTC TTT CCA GAG	TTG AGA ACC TCT GTG TAT TTG
UBC_HHG	GCA CTG GTT TTC TTT CCA GAG	CAC GAA GAT CTG CAT TGT TCA
YWHAZ_HHG	ACT TTT GGT ACA TTG TGG CTT	CCG CCA GGA CAA ACC AGT AT

HHG: housekeeping genes used for normalisation

Table 3.2: Condensed list of all the GO annotations significantly associated with the up-regulated genes at the post-diauxic shift

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0016676	19	6	<0,00	oxidoreductase activity, acting on a heme group of donors, oxygen as acceptor
GO:0016675	19	6	<0,00	oxidoreductase activity, acting on a heme group of donors
GO:0004129	19	6	<0,00	Cytochrome c oxidase activity
GO:0015002	19	6	<0,00	heme-copper terminal oxidase activity
GO:0015078	68	9	<0,00	hydrogen ion transmembrane transporter activity
GO:0015077	86	9	<0,00	monovalent inorganic cation transmembrane transporter activity
…	…	…	…	…
GO:0009055	33	5	0,01	electron carrier activity
GO:0009313	18	4	0,01	oligosaccharide catabolic process
GO:0045333	108	8	0,01	cellular respiration
GO:0009060	85	7	0,01	aerobic respiration
GO:0005985	8	3	0,01	sucrose metabolic process
GO:0005987	8	3	0,01	sucrose catabolic process
GO:0000025	2	2	0,01	maltose catabolic process
…	…	…	…	…
GO:0008645	24	4	0,01	hexose transport
GO:0046906	24	4	0,01	tetrapyrrole binding
GO:0015749	24	4	0,01	monosaccharide transport
GO:0008324	170	9	0,02	cation transmembrane transporter activity
GO:0005746	27	4	0,02	mitochondrial respiratory chain
GO:0015926	27	4	0,02	glucosidase activity

Table 3.2 continued: Condensed list of all the GO annotations significantly associated with the up-regulated genes at the post-diauxic shift

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0006123	12	3	0,02	mitochondrial electron transport, cytochrome c to oxygen
GO:0005751	12	3	0,02	mitochondrial respiratory chain complex IV
GO:0045277	12	3	0,02	respiratory chain complex IV
GO:0042773	28	4	0,02	ATP synthesis coupled electron transport
GO:0042775	28	4	0,02	mitochondrial ATP synthesis coupled electron transport
GO:0006119	29	4	0,02	oxidative phosphorylation
GO:0000023	13	3	0,02	maltose metabolic process
GO:0005215	443	15	0,02	transporter activity
GO:0022857	349	13	0,02	transmembrane transporter activity
GO:0005984	30	4	0,02	disaccharide metabolic process
GO:0022904	30	4	0,02	respiratory electron transport chain
GO:0022900	31	4	0,02	electron transport chain
GO:0070469	32	4	0,02	respiratory chain
GO:0022891	314	12	0,02	substrate-specific transmembrane transporter activity
GO:0005353	15	3	0,02	fructose transmembrane transporter activity
GO:0015578	15	3	0,02	mannose transmembrane transporter activity
…	…	…	…	…
GO:0015146	4	2	0,02	pentose transmembrane transporter activity
…	…	…	…	…
GO:0015145	17	3	0,03	monosaccharide transmembrane transporter activity

Table 3.2 continued: Condensed list of all the GO annotations significantly associated with the up-regulated genes at the post-diauxic shift

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0044455	166	8	0,03	mitochondrial membrane part
GO:0005506	38	4	0,03	iron ion binding
GO:0044724	95	6	0,03	single-organism carbohydrate catabolic process
GO:0044275	39	4	0,03	cellular carbohydrate catabolic process
…	…	…	…	…
GO:0051119	20	3	0,04	sugar transmembrane transporter activity
GO:0008643	43	4	0,04	carbohydrate transport
GO:0016052	105	6	0,04	carbohydrate catabolic process
GO:0009311	44	4	0,04	oligosaccharide metabolic process

Population term: the number of genes in the whole yeast population set (5640 genes) that are annotated to the GO term in question. Study term: the number of genes in the study set that is annotated to the GO term in question. The study set contained 75 significantly up-regulated genes with a log (FC) < 1.5. Wt*DFNA5* transformed yeast cells were used as a reference. GO-enriched terms related to mitochondria are indicated in red, to transporter activity in blue and GO terms related to energy metabolism in green. Adj.p.value, p-value adjusted for multiple hypothesis testing. '… 'denotes a gap in the list due to space restriction.

Table 3.3: List of all the GO annotations significantly associated with the down-regulated genes at the post-diauxic shift

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0006007	52	10	<0,00	glucose catabolic process
GO:0019320	59	10	<0,00	hexose catabolic process
GO:0046365	65	10	<0,00	monosaccharide catabolic process
GO:0006096	32	8	<0,00	glycolysis
GO:0044724	95	10	<0,00	single-organism carbohydrate catabolic process
GO:0006006	98	10	<0,00	glucose metabolic process
GO:0016052	105	10	<0,00	carbohydrate catabolic process
GO:0006094	32	7	<0,00	gluconeogenesis
GO:0019319	33	7	<0,00	hexose biosynthetic process
GO:0046364	34	7	<0,00	monosaccharide biosynthetic process
GO:0019318	115	10	<0,00	hexose metabolic process
GO:0005996	121	10	<0,00	monosaccharide metabolic process
GO:0006091	189	9	<0,00	generation of precursor metabolites and energy
GO:0016051	97	7	<0,00	carbohydrate biosynthetic process
GO:0005886	454	13	<0,00	plasma membrane
GO:0005975	343	11	<0,00	carbohydrate metabolic process
GO:0044723	298	10	<0,00	single-organism carbohydrate metabolic process
GO:0044445	235	9	<0,00	cytosolic part
GO:0016861	8	3	<0,00	intramolecular oxidoreductase activity, interconverting aldoses and ketoses
GO:0005829	531	12	<0.01	cytosol
GO:0022626	168	7	<0.01	cytosolic ribosome

Table 3.3 continued: List of all the GO annotations significantly associated with the down-regulated genes at the post-diauxic shift

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0006098	15	3	<0.01	pentose-phosphate shunt
GO:0071944	650	13	<0.01	cell periphery
GO:0004365	3	2	<0.01	glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity
GO:0044712	491	11	<0.01	single-organism catabolic process
GO:0006740	19	3	0.01	NADPH regeneration
GO:0016860	20	3	0.01	intramolecular oxidoreductase activity
GO:0046496	50	4	0.01	nicotinamide nucleotide metabolic process
GO:0019362	51	4	0.01	pyridine nucleotide metabolic process
GO:0003735	214	7	0.01	structural constituent of ribosome
GO:0006739	22	3	0.01	NADP metabolic process
GO:0051287	25	3	0.01	NAD binding
GO:0044391	229	7	0.01	ribosomal subunit
GO:0045903	6	2	0.01	positive regulation of translational fidelity
GO:0072524	62	4	0.01	pyridine-containing compound metabolic process
GO:0006733	65	4	0.01	oxidoreduction coenzyme metabolic process
GO:1901576	1945	22	0.02	organic substance biosynthetic process
GO:0044711	817	13	0.02	single-organism biosynthetic process
GO:0009058	1973	22	0.02	biosynthetic process
GO:0006732	144	5	0.04	coenzyme metabolic process
GO:1901575	777	12	0.04	organic substance catabolic process
GO:0022625	89	4	0.04	cytosolic large ribosomal subunit

Table 3.3 continued: List of all the GO annotations significantly associated with the down-regulated genes at the post-diauxic shift

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0006090	13	2	0.05	pyruvate metabolic process

Population term: the number of genes in the yeast population set (5640 yeast genes) that are annotated to the GO term in question. Study term: the number of genes in the study set that is annotated to the GO term in question. The study set contained 36 significantly down-regulated genes with a log (FC) < 1.5. Wt*DFNA5* transformed yeast cells were used as a reference. GO-enriched terms related to translation are indicated in red. Adj.p.value, p-value adjusted for multiple hypothesis testing.

Table 3.4: Condensed list of GO annotations significantly associated with mut*DFNA5* genes down-regulated in stationary phase

ID	Pop.term	Study.term	adj.P.Val	Name
…	…	…	…	…
GO:0006696	29	13	<0.01	ergosterol biosynthetic process
GO:0044108	29	13	<0.01	cellular alcohol biosynthetic process
GO:0016129	29	13	<0.01	phytosteroid biosynthetic process
GO:0046165	81	23	<0.01	alcohol biosynthetic process
GO:0006694	39	15	<0.01	steroid biosynthetic process
GO:0016126	39	15	<0.01	sterol biosynthetic process
GO:0071704	3529	388	<0.01	organic substance metabolic process
GO:1901617	89	24	<0.01	organic hydroxy compound biosynthetic process
GO:0008202	50	17	<0.01	steroid metabolic process
GO:0016125	50	17	<0.01	sterol metabolic process
GO:0008204	31	13	<0.01	ergosterol metabolic process
GO:0016128	31	13	<0.01	phytosteroid metabolic process
GO:0009277	97	25	<0.01	fungal-type cell wall
…	…	…	…	…
GO:0090502	47	15	<0.01	RNA phosphodiester bond hydrolysis, endonucleolytic
GO:0006629	308	52	<0.01	lipid metabolic process
GO:0005829	531	79	<0.01	cytosol
GO:0006633	33	12	<0.01	fatty acid biosynthetic process
GO:0009987	4554	471	<0.01	cellular process
…	…	…	…	…

Table 3.4 continued: Condensed list of GO annotations significantly associated with mut*DFNA5* genes down-regulated in stationary phase

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0034641	2046	239	<0.01	cellular nitrogen compound metabolic process
GO:0005783	478	72	<0.01	endoplasmic reticulum
GO:1901070	6	5	<0.01	guanosine-containing compound biosynthetic process
GO:0046037	6	5	<0.01	GMP metabolic process
GO:0044432	379	60	<0.01	endoplasmic reticulum part
…	…	…	…	…
GO:1901137	223	40	<0.01	carbohydrate derivative biosynthetic process
GO:0042175	366	58	<0.01	nuclear outer membrane-endoplasmic reticulum membrane network
GO:0006753	310	51	<0.01	nucleoside phosphate metabolic process
…	…	…	…	…
GO:0005789	353	56	<0.01	endoplasmic reticulum membrane
GO:0030497	4	4	<0.01	fatty acid elongation
GO:0006725	1974	230	<0.01	cellular aromatic compound metabolic process
…	…	…	…	…
GO:0072599	38	12	<0.01	establishment of protein localization to endoplasmic reticulum
GO:0045047	38	12	<0.01	protein targeting to ER
GO:1901135	411	62	<0.01	carbohydrate derivative metabolic process
GO:0005844	33	11	<0.01	polysome
GO:0006913	173	32	<0.01	nucleocytoplasmic transport
GO:0022618	144	28	0.01	ribonucleoprotein complex assembly
GO:0006066	130	26	0.01	alcohol metabolic process

Table 3.4 continued: Condensed list of GO annotations significantly associated with mut*DFNA5* genes down-regulated in stationary phase

ID	Pop.term	Study.term	adj.P.Val	Name
…	…	…	…	…
GO:0043001	11	6	0.01	Golgi to plasma membrane protein transport
GO:0046365	65	16	0.01	monosaccharide catabolic process
…	…	…	…	…
GO:0072659	17	7	0.01	protein localization to plasma membrane
GO:0090002	17	7	0.01	establishment of protein localization to plasma membrane
…	…	…	…	…
GO:0004312	9	5	0.02	fatty acid synthase activity
GO:0009147	9	5	0.02	pyrimidine nucleoside triphosphate metabolic process
GO:0019319	33	10	0.02	hexose biosynthetic process
GO:0070972	45	12	0.02	protein localization to endoplasmic reticulum

Population term: the number of genes in the yeast population set (5640 yeast genes) that are annotated to the GO term in question. Study term: The numbers of genes in the study set that are annotated to the GO term in question. The study set contained 541 significantly up-regulated genes with a log (FC) < 1.5. Mut*DFNA5*-transformed yeast cells in exponential phase were used as a reference. The first 50 GO terms were left out, as they were all related to ribosomal processes and similar to wt*DFNA5* down-regulated processes. GO terms related to lipid metabolism are indicated in red, GO terms associated to the endoplasmic reticulum/protein transport are indicated in green. '… 'denotes a gap in the list due to space restriction. Adj.p.value, p-value adjusted for multiple hypothesis testing.

Table 3.5: Condensed list of GO annotations significantly associated with wt*DFNA5* genes down-regulated in stationary phase

ID	Pop.term	Study.term	adj.P.Val	Name
…	…	…	…	…
GO:0005856	239	34	<0.01	Cytoskeleton
GO:0031974	1070	98	<0.01	membrane-enclosed lumen
GO:0008610	187	29	<0.01	lipid biosynthetic process
GO:0022613	427	50	<0.01	ribonucleoprotein complex biogenesis
GO:0044430	224	32	<0.01	cytoskeletal part
GO:0000226	106	20	<0.01	microtubule cytoskeleton organization
GO:0005874	64	15	<0.01	Microtubule
GO:0005935	166	26	<0.01	cellular bud neck
GO:0071944	650	66	<0.01	cell periphery
GO:0048610	406	47	<0.01	cellular process involved in reproduction
GO:0000922	82	17	<0.01	spindle pole
GO:0022414	254	34	<0.01	reproductive process
GO:0005828	6	5	<0.01	kinetochore microtubule
GO:0005815	75	16	<0.01	microtubule organizing center
GO:0005816	75	16	<0.01	spindle pole body
GO:0044764	122	21	<0.01	multi-organism cellular process
GO:1901617	89	17	<0.01	organic hydroxy compound biosynthetic process
GO:0005880	7	5	<0.01	nuclear microtubule
GO:0032040	48	12	<0.01	small-subunit processome
GO:0046165	81	16	<0.01	alcohol biosynthetic process
GO:0005788	16	7	<0.01	endoplasmic reticulum lumen
GO:0034660	423	46	<0.01	ncRNA metabolic process

Table 3.5 continued: Condensed list of GO annotations significantly associated with wt*DFNA5* genes down-regulated in stationary phase

ID	Pop.term	Study.term	adj.P.Val	Name
GO:0044085	1017	89	<0.01	cellular component biogenesis
GO:0019953	219	29	<0.01	sexual reproduction
GO:0044703	219	29	<0.01	multi-organism reproductive process
GO:0005200	29	9	<0.01	structural constituent of cytoskeleton
GO:0005876	23	8	<0.01	spindle microtubule
GO:0007051	44	11	<0.01	spindle organization
GO:0007052	38	10	<0.01	mitotic spindle organization
GO:0044452	71	14	<0.01	nucleolar part
GO:0071840	2122	158	<0.01	cellular component organization or biogenesis
GO:0007010	232	29	<0.01	cytoskeleton organization
GO:0016538	26	8	<0.01	cyclin-dependent protein serine/threonine kinase regulator activity
GO:0044815	20	7	<0.01	DNA packaging complex
GO:0000027	34	9	<0.01	ribosomal large subunit assembly
GO:0000462	95	16	<0.01	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
GO:0044450	15	6	<0.01	microtubule organizing center part

Population term: the number of genes in the yeast population set (5640 yeast genes) that are annotated to the GO term in question. Study term: The numbers of genes in the study set that are annotated to the GO term in question. The study set contained 331 significantly up-regulated genes with a log (FC) < 1.5. Wt*DFNA5*-transformed yeast cells in exponential phase were used as a reference. First 40 GO terms were left out, as they were all related to ribosomal processes and similar to mut*DFNA5* down-regulated processes. GO terms related to cytoskeleton/microtubuli are indicated in red. Adj.p.value, p-value adjusted for multiple hypothesis testing. '… 'denotes a gap in the list due to space restriction.

Table 3.6: List of biological GO annotations significantly associated with mut*DFNA5* genes up-regulated in HEK293T cells

Biological GO term	Pop.term	Study.term	adj.P.Val	Name
GO:0007275	4169	45	<0.01	multicellular organismal development
GO:0032502	4721	47	<0.01	developmental process
GO:0044767	4669	46	<0.01	single-organism developmental process
GO:0032501	5653	50	<0.01	multicellular organismal process
GO:0044707	5444	49	<0.01	single-multicellular organism process
GO:0051591	86	8	<0.01	response to cAMP
GO:0048856	4195	41	<0.01	anatomical structure development
GO:0048513	2587	31	<0.01	organ development
GO:0048731	3621	37	<0.01	system development
GO:0050794	7865	56	<0.01	regulation of cellular process
GO:0048523	3017	33	<0.01	negative regulation of cellular process
GO:0046683	114	8	<0.01	response to organophosphorus
GO:0009888	1395	22	<0.01	tissue development
GO:0080090	4512	41	<0.01	regulation of primary metabolic process
GO:0048585	948	18	<0.01	negative regulation of response to stimulus
GO:0050789	8324	57	<0.01	regulation of biological process
GO:0048519	3313	34	<0.01	negative regulation of biological process
GO:0014074	129	8	<0.01	response to purine-containing compound
GO:0009893	2193	27	<0.01	positive regulation of metabolic process
GO:0010604	1992	25	<0.01	positive regulation of macromolecule metabolic process
GO:0060255	4267	38	<0.01	regulation of macromolecule metabolic process

Table 3.6 continued: List of biological GO annotations significantly associated with mut*DFNA5* genes up-regulated in HEK293T cells

Biological GO term	Pop.term	Study.term	adj.P.Val	Name
GO:0043407	65	6	<0.01	negative regulation of MAP kinase activity
GO:1902532	287	10	<0.01	negative regulation of intracellular signal transduction
GO:0010605	1464	21	<0.01	negative regulation of macromolecule metabolic process
GO:0043409	111	7	<0.01	negative regulation of MAPK cascade
GO:0006469	166	8	<0.01	negative regulation of protein kinase activity
GO:0031325	2083	25	<0.01	positive regulation of cellular metabolic process
GO:0065007	8823	57	<0.01	biological regulation
GO:0031323	4611	39	<0.01	regulation of cellular metabolic process

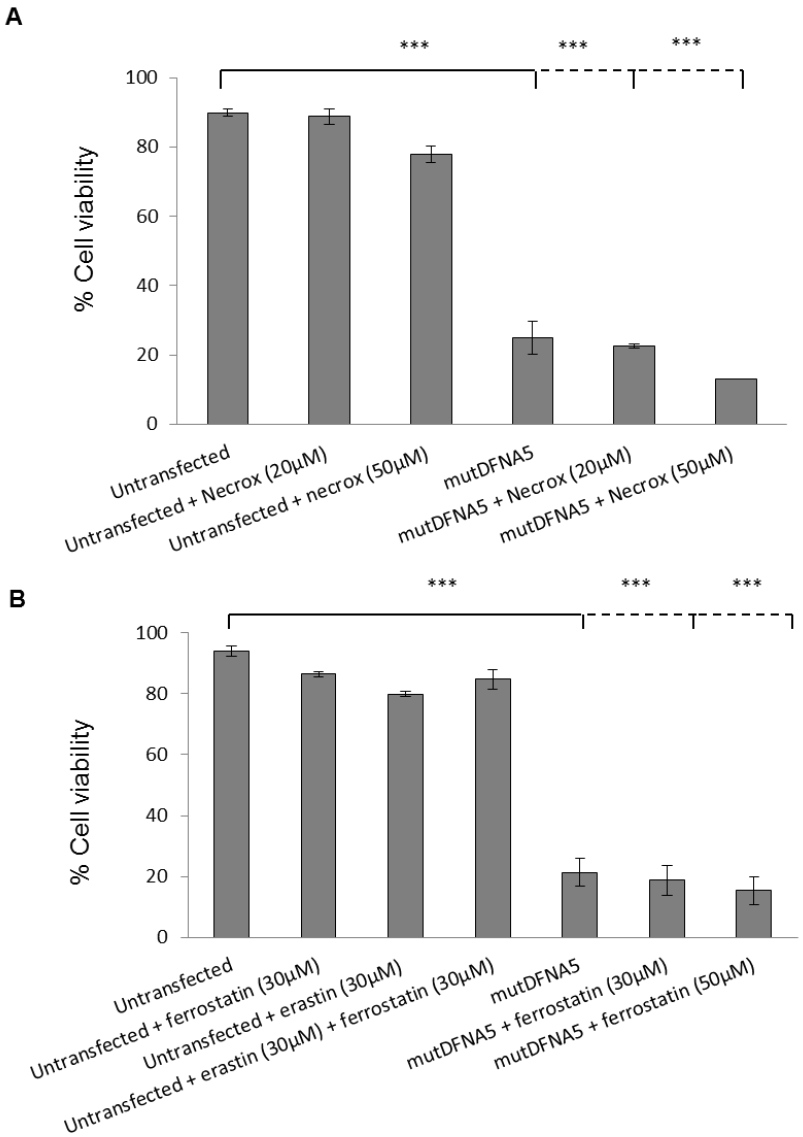
Population term: the number of genes in the human population set (26640 human genes) that are annotated to the GO term in question. Study term: the number of genes in the study set that is annotated to the GO term in question. The study set contained 79 significantly up-regulated genes with a log (FC) < 0.5. Wt*DFNA5*-transfected HEK293T cells were used as a reference. GO terms related to the cAMP response and the MAPK pathway are indicated in red. Adj.p.value, p-value adjusted for multiple hypothesis testing.

Table 3.7: List of biological GO annotations significantly associated with mut*DFNA5* genes down-regulated in HEK293T cells

Biological GO term	Pop.term	Study.term	adj.P.Val	Name
GO:0006986	134	5	<0.01	response to unfolded protein
GO:0035966	142	5	<0.01	response to topologically incorrect protein

Population term: the number of genes in the human population set (26640 human genes) that are annotated to the GO term in question. Study term: the number of genes in the study set that is annotated to the GO term in question. The study set contained 50 significantly up-regulated genes with a log (FC) < 0.5. Wt*DFNA5*-transfected HEK293T cells were used as a reference. Adj.p.value, p-value adjusted for multiple hypothesis testing.

V.3 Supplementary Material Chapter IV



Suppl. Fig. 4.1: Role of Necrox and ferrostatin on mutDFNA5 cell viability. a. 20 µM or 50 µM Necrox was added overnight for 16 h to mut*DFNA5* transfected and untransfected HEK293T cells. Cell viability was measured the following day by flow cytometry using PI as a cell death marker. **b.** 30 µM or 50 µM ferrostatin and/or erastin (positive control) was added overnight for 16 h to mut*DFNA5* transfected and untransfected HEK293T cells. Cell viability was measured the following day by flow cytometry using PI as a cell death marker. * denotes a significant difference in cell viability in mut*DFNA5* compared to untransfected HEK293T cells. ***: p<0.001.

VI. Dankwoord

Vijf jaar, een hele periode waarin ik hoogtes en dieptes heb gekend, en een hoofdstuk dat nu afgesloten wordt. Gedurende deze periode heb ik vele nieuwe mensen ontmoet die het mogelijk maakten om dit project tot een goed einde te brengen.

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